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Development and application of procedures to detect low molecular mass enzymes and evaluation of a zymogram method for microbial endoglucanases

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Development and application of procedures to detect low molecular mass enzymes and evaluation of a zymogram method for microbial endoglucanases

by

Scott Michael Holt

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GENERAL INTRODUCTION

This dissertation is comprised of two parts. Each part consists of an introduction, literature review, materials and methods, results, and discussion. A general summary of both parts and a literature cited section follows the discussion section of part 2. Isolation, purification, and characterization of microbial enzymes that can degrade cellulosic material has been hampered by their complex nature. In this study, a zymogram method was adapted for the identification cellulose and xylan-degrading enzymes from mesophilic microorganisms. Part 1 of this dissertation is titled "Development and application of procedures to detect low molecular mass enzymes." Procedures to detect enzymes that could penetrate ultrafiltration membranes with a rated 10 kDa exclusion were developed. By using a denaturing zymogram method in combination with SDS-polyacrylamide gel electrophoresis, molecular masses of a cellulase and a xylanase that passed through an ultrafilter were determined. The second part of this dissertation is titled "Evaluation of a zymogram method to for microbial endoglucanases." The zymogram method used in part 1 was evaluated for its ability to detect endoglucanase molecular forms produced by *Trichoderma reesei* QM 9414, *Myrothecium verrucaria* ATCC 9095, and *Bacillus subtilis* PAP 115.

PART I: DEVELOPMENT AND APPLICATION OF PROCEDURES TO DETECT LOW MOLECULAR MASS ENZYMES

INTRODUCTION

The objectives of this research were to develop methods to detect enzymes with a molecular mass less than 10 kDa and to screen environmental isolates and commercial enzyme preparations for the presence of very small enzymes. Enzymes of less than 10 kDa in size have been reported in the literature (Ganju et al., 1989; Whitney et al., 1969); however, these discoveries were accidental, not targeted. The purpose of the studies conducted by these investigators was to characterize some enzyme preparations, not to isolate and identify low molecular mass enzymes. Small enzymes that are simple in structure would facilitate basic research involved with the elucidation of structure-function relationships (Egami et al., 1969). Low molecular mass enzymes could also be used as labels for probes in diagnostic bioassays. Enzyme labels are used to indicate that binding between probe and target molecules has occurred. Binding is signaled by the catalytic action of the enzyme on a substrate(s); detectable end-products are released (Blake and Gould, 1984). Although the use of enzymes would be preferred over radioisotopes in diagnostic assays, enzyme-labeled probes often lack sensitivity (Blake and Gould, 1984). Biotin/avidin (Kendal et al., 1983) and redox-cycling (Johannsson et al., 1986) amplification

methods, for example, have increased detection sensitivity of enzyme-based assays. The large molecular mass (40 kDa to 540 kDa) of enzyme labels currently used, however, may theoretically limit signal magnification (Walker et al., 1992). Large molecules (95 to 150 kDa in size) also may sterically interfere with the interaction between probe and target molecules, resulting in limited signal production (Kronick and Grossman, 1983; Walker et al., 1992). The use of small enzyme labels of 10 kDa or less in size could enhance signal response by minimizing interferences between probe and target molecules. In addition, a large molecular mass enzyme, such as *E. coli* ß-galactosidase (540 kDa), physically limits the number of label molecules that can be coupled per probe molecule compared to radioisotopes (Walker et al., 1992) or a very small enzyme. More small enzyme label per probe molecule could increase the end-product signal.

In this study, microorganisms, commercial enzyme preparations, and mammalian tissue samples were screened for the presence of low molecular mass enzymes. Enzyme samples were subjected to ultrafiltration through membranes that exclude proteins 10-kDa or larger in size. The ultrafiltrates should contain a relatively small proportion of components (proteins) that exceed 10 kDa in size. Retentates and ultrafiltrates were assayed for enzyme

activity by radial gel diffusion assays using twenty different substrates. Fifteen of 257 samples were ultrafiltrate-positive for CMCase or xylanase activities. These 15 samples were the only samples in which a low molecular mass enzyme was detected. Two of the ultrafiltrate-positive samples were subjected to denaturing SDS-polyacylamide gel electrophoresis and zymogram analysis to determine the molecular mass of enzyme components that penetrated the membrane.

LITERATURE REVIEW

Applications of Low Molecular Mass Enzymes

Low molecular mass enzymes (<10 kDa in size) would have applications in basic research and as labels for antibody, antigen, or nucleic acid probes in diagnostic assays. The study of very small natural enzymes that are simple in overall conformation may facilitate interpretation of structure-function relationships and help gain a better understanding of functional properties such as thermostability and the effects of pH on enzyme activity.

Small enzymes also could be used as labels for diagnostic probes such as antibodies, antigens or nucleic acids. In a standard immunoassay or hybridization test, enzyme-labeled probes (conjugates) would signal binding of antigen to antibody (Blake and Gould, 1984) or hybridization of nucleic acid to target nucleotide sequences (Dahlen et al., 1989). The binding signal is generated by enzyme action on a substrate to release readily detectable end-products.

Radioisotopes are frequently used as labels for diagnostic assays because radioactivity can de detecetd with great sensitivity and ususally does not interfere with the interaction between probe and target molecules (Blake

and Gould, 1984). The use of radioactive labels, however, has drawbacks that include stringent regulations governing their handling, possible health risks, and relatively short half-lives of some radioisotopes (Walker et al., 1992). Although fluorescent labels, such as fluorescein and lanthanide chelates, have been used in bioassays, their applicability is limited because the antibody conjugation procedure causes loss of avidity (Phillips et al., 1987) and special equipment is needed for signal quantification (Dahlen et al., 1989).

In contrast, enzyme labels pose no health risks due to radiation, do not require stringent regulations governing their use, have long shelf lives (with proper storage), and the formation of readily determined end-product signals allows qualitative assessment of results, often without the need for special equipment (Blake and Gould, 1984; Walker et al., 1992). Nevertheless, enzyme labels are often less sensitive than radioisotope-based assays (Blake and Gould, 1984), and because of their relatively large molecular size (40 to 540 kDa), may sterically interfere with binding between probe and target (Walker et al., 1992).

Various amplification methods have been developed to increase the sensitivity of enzyme labels in diagnostic assays (Johannsson et al., 1986; Kendal et al., 1983). The biotin/avidin amplification system was originally developed

by Bayer and Wilchek (1974) for immunochemical staining of tissues. The avidin molecule has a natural affinity for biotin which is the basis for numerous diagnostic strategies using antibody, antigen, and nucleic acid probes (Wilchek and Bayer., 1988). For example, one strategy is to couple biotin to target molecules which can subsequently be detected using avidin probes that were conjugated to enzyme labels. Binding between biotin and avidin is signaled by enzyme action on a specific substrate. By using biotin/avidin technology, Kendall et al. (1983) increased the detection sensitivity by as much as 4134-fold over a commercial radioimmunoassay for hepatitis B surface antigen. The increased sensitivity was attributed to the high affinity interaction between avidin and biotin which forms a complex that is stable during rigorous immunoassay conditions. In addition, each avidin molecule has four binding sites for biotin. As stated by Kendall et al. (1983), "theoretically it is possible to couple more biotinylated enzyme per avidin molecule than enzyme per antibody molecule without a concomitant loss of specific binding activity." Although the biotin/avidin system has been used for a variety of bioanalyses, two main limitations are associated with its application (Wilchek and Bayer, 1988). First, excessive nonspecific binding of avidin is often observed; This nonspecific binding is caused by the

presence of carbohydrate chains on the molecule that can bind with other proteins. Nonspecific binding can be reduced by replacing egg-white avidin with nonglycosylated bacterial strepavidin. Secondly, although biotin is a small molecule (244 Da), avidin has a molecular mass of 66 kDa which may sterically interfere with probe:target interactions, as described by Walker et al. (1992).

Another amplification method was described by Self (1985) who developed an enzymatic cycling reaction for magnifying the signal produced by alkaline phosphatase. Alkaline phosphatase from calf intestine has a molecular mass of 100 kDa and is commonly used as an enzyme label for immunoassays and oligonucleotide probes (Blake and Gould, 1984; Dahlen et al., 1989). The Self (1985) cycling reaction was initiated by dephosphorylation of the cofactor NADP to produce NAD by the action of alkaline phosphatase. Dephosphorylation activates an NAD-NADH redox-cycle involving the enzymes alcohol dehydrogenase and diaphorase. Alcohol dehydrogenase oxidize ethanol to acetaldehyde and in the process change NAD to NADH. A colored product is generated by reduction of a tetrazolium salt by diaphorase which also oxidizes NAD back to NADH. The cycling amplification system reportedly is more sensitive than some conventional enzyme and radioisotope-based immunoassays (Joannsson, et al., 1986).

The sensitivity of enzyme-based assays has also been increased by using bioluminescent, chemiluminescent, and fluorescent substrate systems whose hydrolysis products are measurable at lower concentrations than visibly colored products (Coutlee et al., 1989; Seitz, 1984).

In addition to alkaline phosphatase, enzymes that are commonly used as labels for bioassays include *E. coli* ß-galactosidase (540 kDa) and horseradish peroxidase (40 kDa; Walker et al., 1992). These enzyme labels are relatively large molecules compared with the sizes of IgG antibody (150 kDa), $F(AB)'_2$ IgG antibody (95 kDa), and some synthetic oligonucleotide probes (21-26 nucleotides; Jablonski et al., 1986; Li et al., 1987) to which they might be attached. Large molecular mass labels have the potential to sterically interfere with the interaction between probe and target molecules (Avrameas et al., 1978; Krontic and Grossman, 1983; Walker et al., 1992). Additionally, a large molecular mass enzyme, such as *E. coli* ß-galactosidase (540 kDa), physically limits the number of label molecules that can be coupled per probe molecule compared to radioisotope labels (Walker et al., 1992) or a very small enzyme. Evidence that steric hindrance can lower the detection sensitivity of bioassays was reported by Kronick and Grossman (1983), who developed an immunoassay using a fluorescent phycobiliprotein (240 kDa) antibody conjugate. The

phycobiliprotein conjugate displayed a 12-fold signal enhancement over a fluorescein conjugate; however, only a 6-fold enhancement was achieved in the actual immunoassay. The possibility that ineffective antibodies inhibited immunoassay signal enhancement was minimized by saturating accessible antigen binding sites with the phycobiliprotein-antibody conjugate. Kronick and Grossmen (1983) concluded that the physical size of the phycobiliprotein blocked access of some binding sites on the antibody conjugate to corresponding binding sites on the antigen.

Low molecular mass enzymes (<10 kDa) could increase detection sensitivity of diagnostic assays by eliminating steric interferences caused by large enzyme labels. Small enzymes may also facilitate signal amplification because more label could theoretically be attached per probe molecule compared to commonly used enzymes, such as alkaline phosphatase, ß-galactosidase, and peroxidase.

Low Molecular Mass Enzymes Described in the Literature

Lysozyme

Lysozymes have been isolated from a variety of animal, plant and microbial species (Osserman et al., 1972). Most lysozymes are between 15 to 30 kDa in size, for example, hen egg white lysozyme has a molecular mass of 14.6 kDa and is a compact molecule with an ellipsoidal shape (Stryer, 1989).

Lysozyme action breaks the glycosidic bond between the n-acetyl-D-glucosamine and n-acetylmuramic acid units that comprise the peptidogylcan cell wall layer of many microorganisms. The cell wall of *Micrococcus lysodiekticus* is extremely susceptible to lysozyme and has been used as a substrate to assess enzyme activity. Lysozyme action on a suspension of ultravilet-killed and lyophilized *M. lysodeikticus* cells results in turbidity reduction which is measured by detecting changes in light transmission (Rubenstein et al., 1972). Lysozyme has been used as a label in an immunoassay, however, the turbidity reduction assay did not produce a visibly distinct end-product "signal" which limits its sensitivity (Parry et al., 1965; Rubenstein et al., 1972). Although colorimetric (Osawa, 1966; Osawa and Nakazawa, 1966) and fluorimetric (Yang and Hamaguchi, 1980) synthetic substrates have been used to assess lysozyme action, hydrolysis rates were not adequate for bioassay adaptation (Holt and Hartman, unpublished results; Osawa, 1966; Osawa and Nakazawa, 1966).

Ribonucleases

Ribonucleotide-degrading enzymes (RNases) from a wide variety of microorganisms have been isolated and characterized. Many have molecular masses in the range of 10 to 13 kDa (Egami and Nakamura, 1969). Jeffries et al. (1957) developed a method for detecting microbial RNase

activity on a 1% agar medium incorporated with 0.2% yeast RNA as the substrate. Discrete microbial colonies in mixed cultures were screened for RNase production by culturing dilutions of environmental samples on the RNA-agar test medium supplemented with 0.5% glucose and a basal salts solution. After incubation for 24 hours at 35°C, RNase activity was detected by flooding the agar medium with 1N HCl which precipitated unhydrolyzed RNA. Degraded RNA in the agar medium was not precipitated by the 1N HCl treatment, thus revealing zones of clearing around microbial colonies that possessed RNase activity. RNase activity in agar medium can also be detected by staining with a 0.1% ethidium bromide solution and visualizing the nonfluorescent hydrolytic zones with a short-wave UV transilluminator (Schill and Schumacher, 1972).

Cellulases and xylanases

There are numerous reports of cellulases and xylanases produced by microorganisms that range from 2.2 to 12.5 kDa in size (Berghem et al., 1976; Bhat et al., 1988; Grabski and Jeffries, 1991; Whitney et al., 1969; Wong et al., 1986). Cellulases and xylanases degrade cellulose and xylan into soluble products that can be easily metabolized by many bacteria and fungi (Reese, 1977). Most of the small cellulases (2.2 to 12.5 kDa in size) described in the literature were isolated from fungal culture filtrates.

Molecular masses were determined by gel filtration chromatography (Berghem et al., 1976; Bhat et al., 1988; Whitney et al., 1969). For example, Bhat et al. (1988) identified a cellulase component from *Penicillim pinophilum* that was assigned a molecular mass of 10.4 kDa using gel filtration. Further examination of the enzyme by denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE), however, indicated that the molecular mass of the protein was 25 kDa. Bhat et al. (1988) suggested that the large discrepancy between molecular mass estimations observed with gel filtration and SDS-PAGE may be related to the shape of the enzyme. Similar observations dealing with inconsistent molecular mass estimations of xylanases have been reported from *Streptomyces roseiscleroticus* (Grabski and Jeffries, 1991) and *Trichoderma harzianum* (Wong et al., 1980). Grabski and Jeffries (1991) isolated a xylanase from *S. roseiscleroticus* that penetrated an ultrafilter with a 10 kDa membrane exclusion and was determined to be 5 kDa in size using gel filtration. The molecular mass was confirmed using a different gel filtration support matrix; therefore, the possibility that protein adsorption caused an artificially low molecular mass estimate was minimized. Adsorption to the gel filtration matrix could cause the xylanase to exhibit an elution profile that mimicks a lower molecular mass protein. Denaturing SDS-PAGE analysis of the

xylanase, however, indicated that it was 22.6 kDa in size. Grabski and Jeffries (1991) believed that the xylanase might possess an "elongated" or "tapered" molecular shape that facilitated its penetration through pores in the gel matrix, resulting in an elution profile that resembled a lower molecular mass component. An elongated or tapered molecular shape would also aid passage of the protein through pores in an ultrafiltration membrane (Cheryan, 1989). The ability of an enzyme to penetrate small pores, such as those in the cellulose-hemicellulose-lignin matrix of wood, would be advantagous to the microorganism for efficient substrate degradation (Grabski and Jeffries, 1991; Reese, 1977).

Wong et al. (1986) identified three xylanases from *T. harzianum* E58 culture filtrates that passed through polysulfone ultrafilters with a 10 kDa exclusion, even though their molecular masses were determined to be 20, 22, and 29 kDa, by using denaturing SDS-PAGE. These investigators were uncertain if passage through the ultrafilter was attributed to the compact shape of the xylanases or to pore size discrepancies in the membrane. At least two other xylanases and ß-xylosidases were present in the culture filtrate that did not pass through the ultrafiltration membrane. The two xylanases that remained in the ultraretentate exhibited different functional properties than the three xylanases that penetrated the

membrane.

Other enzymes

Other low molecular mass enzymes reported in the literature include an acid phosphatase from bovine liver (11.4 kDa; Millipore Technical Bulletin, 1989), an α-glucosidase from *Bacillus cereus* (12.0 kDa; Yamasaki and Suzuki, 1974), a penicillinase from an alkalophilic *Bacillus* strain (12.5 kDa; Sunaga et al., 1979), and a protease from a myxobacter (8.7 kDa; Ensign and Wolfe, 1966).

Ultrafiltration

Ultrafiltration is a pressure-driven membrane filtration process that has been used for the concentration and separation of materials based on molecular size and shape (Cheryan, 1989; Amicon Catalog, 1989). Theoretically, substances larger than the pore size (referred to as molecular mass cut-off in kDa) of the membrane are retained at the filter surface and become concentrated as ultrafiltration proceeds. Substances smaller than the pore size of the membrane pass through the filter and are separated from components that are retained.

Most ultrafilters are asymmetric membranes that consist of a thin "skinned" surface and a sublayer matrix (Matteson and Orr, 1987). Asymmetry refers to the membrane

pore size which changes from its smallest diameter at the skinned surface to larger diameters throughout the subsurface matrix (Cheryan, 1986). The asymmetric design allows retention of components on the membrane surface instead of within the body of the filter which prevents "plugging". Microporous filters usually have pore sizes that are uniform throughout the body of the membrane; therefore, particles with the same size as the pores could become trapped within the filter matrix and interfere with filtration (Cheryan, 1986).

Cellulose and polysulfone are the most common polymers used for the manufacture of ultrafiltration membranes. The polysulfone membranes have a wider range of pH and temperature tolerance than the cellulose-based filters. In addition, cellulose-based ultrafilters, such as cellulose acetate, are susceptible to the action of microbial cellulases which destroy membrane integrity (Cheryan, 1986).

Disc, hollow fiber, and spiral membrane configurations have been used for ultrafiltration processes (Matteson and Orr, 1987; Amicon Catalog, 1989). Disc ultrafiltration membranes are used with small-volume devices, plate-and-frame or thin-channel modules, and stirred cell units. Small-volume ultrafiltration devices are used for concentration and separation of samples in the microliter to milliliter range (Amicon Catalog, 1989). Small-volume

ultrafiltration devices are convenient to use because they can be otained from the manufacturer as preassembled units that require centrifugation instead of gas pressure. Stirred cell ultrafiltration units are used for sample volumes upward of several milliliters and prevent concentration polarization at the membrane surface by gentle impeller agitation. Concentration of retained solute at the membrane surface can decrease ultrafiltration flow rate (Cheryan, 1986). Plate-and-frame (Matteson and Orr, 1987) or thin-channel modules (Amicon Catalog, 1989) consist of alternately stacked membranes and spacers that are bolted together with end plates. A network of thin channels in the spacers carry sample feed over the membrane surface in a cross-flow orientation. Ultrafiltrate is collected within the membrane matrix and flows toward an outlet (Matteson and Orr, 1987). Cross-flow filtration minimizes concentration polarization by rapid circulation of sample feed over the membrane surface. Assembly and channel cleaning is technically simple but time consuming. Hollow fiber and spiral membrane systems combine the advantage of cross-flow filtration with an increase in membrane surface area per module volume (Matteson and Orr, 1987). Hollow fiber and spiral membrane units are easy to use because they are preassembled and compact in shape compared to plate-and-frame models. All the ultrafiltration devices described,

except the small-volume units, require an external source of pressure to drive the system.

The main advantage of using ultrafiltration over other concentration and separation methods is attributed to the mild conditons (pressure) employed during sample processing which can minimize loss of biological activity (e.g. enzyme activity).

Radial Diffusion Enzyme Assays

Radial diffusion assays in agar gels have been used by microbiologists to assess antibiotic resistance (Brownlee et al., 1949), bacteriophage specificity (Gillies, 1978), colicin potentcy (Milch, 1978), and enzyme activity (Schill and Schumacher, 1972). Enzymes detected by radial diffusion methods include amylases (Iyer and Karthiayana, 1964), glucuronidases (Dahlen and Linde, 1973), glucanases (Teather and Wood, 1982), lysozyme (Gosnell, et al., 1975), nucleases (Jeffries et al., 1957), phosphatases (Satta et al., 1979), and xylansases (Biely et al., 1985). Hartman (1968) summarized early developments in radial diffusion assays. In a standard radial diffusion assay for enzyme activity, substrate is incorporated into an agar medium and enzyme samples are added to wells punched in the gel (Schill and Schumacher, 1972) or applied to the surface in saturated

paper disks (Hartman, 1968). During incubation, samples diffuse radially from their initial point of application and enzymatic activity is detected by the formation of clearing zones (Teather and Wood, 1982) or by the release of colored (Satta et al., 1979) or fluorescent (Dahlen and Linde, 1972) products in the agar. Activity zone size is primarily dependent on agar concentration, incubation temperature, enzyme concentration, and molecular properties of the active component (e.g. molecular size). Activity zones can be quantified by measuring the zone diameter (mm) under standard conditions, such as agar concentration, incubation time and incubation temperature. An estimate of enzyme activity is obtained from a reference curve that was prepared by plotting zone diameter versus concentration of standard enzyme solutions on a semilogarithmic graph (Schill and Schumacher, 1972).

Some enzyme activities have been detected in substrate-laden agar media through the catalytic release of colored (Satta et al., 1979) or fluorescent (Dahlen and Linde, 1972) products. Colorless p-nitrophenol (PNP) or umbelliferone substrate derivatives liberate chromophoric and fluorescent products after enzymatic cleavage, respectively. Colored or activity zones of PNP formation are easily detected against clear and colorless agar media. The umbelliferone fluorescence reaction must be visualized with the aid of a

long-wave (366 nm) ultraviolet light.

Enzymes that act on polymeric substrates are detected in substrate-agar media by the formation of clearing zones resulting from depolymerase action (Gosnell et al., 1975; Durand et al., 1972). Lysozyme activity has been detected in an agar medium containing UV-killed and lyophilized cells of *Micrococcus lysodeikticus* (Gosnell et al., 1975). Hen egg white lysozyme concentrations of 5 ug per 100 ml were detected after incubation for 3 hours at 47°C. Proteases can be detected in a similar fashion by embedding an agar medium with 0.1% casein (Durand et al., 1972). Staining is required for some substrates to enhance contrast of the enzymatic clearing zones (Iyer and Karthiayana, 1964; Teather and Wood, 1982). For example, agar media incorporated with carboxymethylcellulose (CMC) can be stained with 0.1% congo red to detect carboxymethylcellulase hydrolytic zones (Teather and Wood, 1982). Congo red has an affinity for ß-glucans, such as CMC (Teather and Wood, 1982). MacKenzie and Williams (1985) detected 1 ng of purified CMCase protein by using the congo red activity stain procedure. In another example, Iyer and Karthiayana (1964) detected amylase activity in agar medium containing 0.1% soluble starch by inverting the gel over I_2 crystals until clear activity zones were revealed against a dark brown background.

Radial diffusion assays possess several advantages over other screening methods. First, because detection can be accomplished directly on the plate on which the microorganism is isolated, numerous samples can be examined with a minimum of labor. Furthermore, a variety of different enzymes can be examined simultaneously by using the same procedure involving agar media containing different substrates; the assays are convenient, rapid, and inexpensive. Small volumes (2 μL; Schill and Schumacher, 1972) can be assayed. Last, the sensitivity can be very high (1 ng CMCase protein detected, MacKenzie and Williams, 1985).

Zymogram Detection of Enzymes

Zymogram analysis has been used to detect enzyme activity of protein components after isoelectric focusing (Sprey and Lambert, 1985) and denaturing (Schwarz et al., 1987) or nondenaturing (Bartley et al, 1985) gel electrophoresis. The use of denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with zymogram analysis combines the advantages of enzyme identification with molecuar mass estimation (Lacks and Sprinhorn, 1980). A description of zymogram analysis and SDS-PAGE is presented in section II, under the heading "Literature Review".

MATERIALS AND METHODS

Ultrafilter Passage Tests

Samples of purified proteins with known molecular masses were each subjected to ultrafiltration (Ultra-Free MC PTGC polysulfone membrane, 10 kDa exclusion, 0.4 ml, Millipore Corp, Milford, MA) in 400-µl volumes at 1 mg/ml in 0.05 M Tris-HCl buffer, pH 6.8. A crude xylanase preparation from a *Chainia* sp. was filtered in 400-µl volumes at 2 mg/ml of the same buffer. Ultrafiltration was effected by centrifugation (3,000 x g) at room temperature until 400 µl was collected in the ultrafiltrate. Proteins used in the challenge test were *E. coli* ß-galactosidase (540 kDa), bovine serum albumin (66 kDa), soybean trypsin inhibitor (20 kDa), hen egg white lysozyme (14.4 kDa), cytochrome c (12.5 kDa), and a *Chainia* sp. xylanase (5 kDa). The xylanase was obtained from Dr. Peter Reilly (Department of Chemical Engineering, Iowa State University, Ames, IA) as a powdered crude extracellular enzyme preparation. The presence of bovine serum albumin, trypsin inhibitor, and cytrochrome c in the ultrafiltrates were determined by using the dye-binding protein assay described later. The presence of ß-galactosidase, lysozyme, and xylanase activities in the ultrafiltrates were determined qualitatively by radial

diffusion as described under "Protein and Enzyme Activity Measurements". Lysozyme activity was detected in petri dishes containing 0.1% (w/v) lysozyme substrate (Difco), 0.02% (w/v) sodium azide, and 0.1 M sodium phosphate buffer in 1.0% agar, pH 6.8. Lysozyme substrate consisted of freeze-dried *Micrococcus lysodiektikus* cells. Clearing zones of cell lysis were detected against a turbid background after incubation for 24 hours at 37°C (Gosnell et al., 1975).

Isolation of Microorganisms

Culture media were from Difco (Detroit, MI). Soil samples, obtained from central Iowa locations, were suspended (1 g) in 99-ml of 0.1% peptone buffer, mixed, and serially diluted (1 ml) in 99-ml dilution blanks. Dilutions (0.1 ml) were cultured on Trypticase-Soy and Malt-Extract agars to isolate bacteria and fungi. Trypticase-Soy agar cultures were incubated for 24 hours at 37°C and Malt-Extract agar cultures were incubated for 48 hours at 30°C. Isolated colonies were purified by subculturing onto Trypticase-Soy agar or Malt-Extract agar and incubated for the appropriate times and temperatures. Colonies of the purified isolates were subcultured onto Trypticase-Soy or Malt-Extract agar slants (16 mm screw-cap test tubes) and

incubated for 24 hours (bacteria) or five days (fungi). Agar slants were prepared in duplicate. One slant was stored at 4°C and the other was used as inoculum for enzyme induction broth. Fungal cultures obtained from Dr. Paul A. Hartman (Department of Microbiology, Immunology, and Preventive Medicine, Iowa State University, Ames, IA) included *Aspergillus niger* ATCC 9029, *Phanerochaete chrysosporium* NRRL 6359 and ME-446, *Phlebia tremellosus* PRL 2845, PRL 2901, and PRL 2902, and *Pleurotus ostreatus* FP-101798-Sp. and NRRL 2366. Fungal cultures obtained from the American Type Culture Collection (ATCC) included *Aspergillus fumigatus* ATCC 46324, *Diplodia gossypina* ATCC 26123, *Myrothecium verrucaria* ATCC 9095, and *Trametes versicolor* ATCC 12679.

Screening Procedure for Low Molecular Mass Enzymes

The microbial isolates, including all fungul cultures, were suspended in 5.0 ml of 0.1% peptone buffer and mixed. Then either 0.5 or 5.0 ml of the suspension was inoculated into 1-L Erlenmyer flasks containing 200 ml of enzyme induction broth. The enzyme induction broth used for bacterial isolates consisted of Nutrient Broth supplemented with 0.1% cellobiose, 0.1% dextran, 0.1% malt extract, 0.1% pectin, 0.1% soluble starch, 0.1% xylan and 0.5% yeast

extract. The pH of the medium was 6.8 after sterilization in an autoclave. The enzyme induction broth used for fungal isolates consisted of 10 g cellulose, 3.8 g $NaNO_3$, 2.0 g $NaH_2PO_4 \cdot H_2O$, 1.5 g Na_2HPO_4, 1.0 g glucose, 0.6 g NH_4NO_3, 0.3 g $MgSO_4 \cdot H_2O$, 0.2 g KH_2PO_4, 0.1 g K_2HPO_4, 2.0 mg $ZnSO_4 \cdot 7H_2O$, 1.2 mg $Ca(NO_3)_2 \cdot 4H_2O$, 0.4 mg $CoCL_2 \cdot 6H_2O$, 0.2 mg $CuSO_4 \cdot 5H_2O$, 0.08 mg $MnCl_2 \cdot 4H_2O$, 0.06 mg H_3BO_3, 0.05 mg $FeSO_4 \cdot 7H_2O$, and 0.04 mg $(NH_4)_6Mo_7O_{24}$ per liter of dH_2O. The final pH of the medium was 6.6 after sterilization in an autoclave.

The bacterial isolates were incubated at 200 rpm in a reciprocating water bath (Orbit Microprocessor Shaker Bath, Lab-Line Instruments Inc. Melrose Park, IL) for 24 hours at 35°C. The fungal isolates were incubated in static cultures for 5 days at 30°C. After incubation, 0.5 ml of each bacterial culture was pelleted by centrifugation at 10,000 x *g* (Micro-Centaur Centrifuge, MSE Scientific Instruments, Sussex, England) for 5 minutes at room temperature. The supernatent was collected and the pellet was resuspended in 1.0 ml of lysis buffer that consisted of 0.025 M Tris-HCl, pH 7.0, 8% sucrose, 50 mM EDTA, 0.1% Triton X-100, 2.5 mg DNase, 2.5 mg RNase, 2 mg lysozyme, 0.7 mg lysostaphin, and 20 μg of mutanolysin. The enzymes were added to the lysis buffer from stock solutions in 0.025 M Tris-HCl buffer, pH 7.0. The enzyme stock solutions consisted of 10 mg/ml RNase, 10 mg/ml DNase, 10 mg/ml lysozyme, 17 mg/ml

lysostaphin, and 1 mg/ml mutanolysin. The lysis buffer was similar to that described by Kaspar et al. (1987) except that the 0.1 M phosphate buffer was replaced with 0.025 M Tris-HCl, pH 7.0, and lysostaphin and mutanolysin were added. The enzyme stock solutions were stored frozen at -100°C until needed. The bacteria in lysis buffer were incubated at 35°C for 30 minutes. Any remaining intact cells and cellular debris were sedimented by centrifugation at 10,000 x g (room temperature), and 100 µl of each supernatent was mixed with 300 µl of 0.025 M Tris-HCl buffer, pH 7.0. The diluted supernatents (400 µl) were subjected to ultrafiltration (Ultra-Free MC PTGC polysulphone 0.4 ml ultrafiltration units, 10 kDa exclusion, Millipore Corp., Bedford, MA) using centrifugation at 3,000 x g (room temperature) until 200 µl of ultrafiltrate was collected. This procedure usually took about 30 minutes. Enzyme activities were detected in the lysates, retentates, and ultrafiltrates by radial diffusion in substrate-agar plates. The Ultra-Free MC PTGC (10 kDa exclusion) units were used in all experiments involving ultrafiltration.

One milliliter from each fungal culture was clarified by centrifugation for 5 minutes at 10,000 x g, and 400 µl was filtered as described for the bacterial samples. Centrifugation and ultrafiltration were performed at room temperature. Retentates and ultrafiltrates were assayed for

enzyme activity by the radial diffusion method.

Powdered commercial enzyme preparations (5 mg) were suspended in 1 ml of 0.1 M Tris-HCl buffer (pH 7.0), clarified by centrifugation at 10,000 x g for 5 minutes, and filtered as described for the bacterial samples. Retentates and ultrafiltrates were assayed for enzyme activity by the radial diffusion method.

Bovine, chicken, and swine livers were obtained from an Ames, Iowa grocery. Two-hundred and fifty grams of each liver sample was washed with 1 L of 0.9% NaCl to remove extraneous blood and then was homogenized in a Waring blender at high speed in 250 ml of 0.1 M Tris-HCl (pH 7.6) containing 100 mM NaCl, 1 mM $MgCl_2$, 0.02 mM $ZnSO_4$, 1 mM $CaCl_2$, and 1 mM $MnCl_2$ (Hua et al., 1985). The homogenized tissue samples were clarified by centrifugation (10,000 x g) at 4°C for 30 minutes. The clarified supernatents were diluted 4-fold with the Tris-HCl buffer, ultrafiltered, and enzyme activities were detected as described for the bacterial isolates.

Protein and Enzyme Activity Measurements

All materials except for culture media were obtained from Sigma Chemical Co. (St Louis, MO), unless indicated otherwise. The culture media were purchased from Difco.

Protein concentration was determined by using a Coomassie Brilliant Blue G-250 dye-binding assay (Bio-Rad Protein Assay, Bio-Rad, Richmond, CA). Bovine serum albumin was used as the protein standard.

Enzyme activity was qualitatively determined by radial diffusion (Schill and Schumacher, 1972) in substrate-containing agar media and quantitatively determined using digest assays. The radial diffusion media contained 1% agar, buffer, 0.02% sodium azide (except when determining peroxidase activity), and an appropriate substrate. 4-methylumbelliferone substrate derivatives were used at 50 μg/ml of buffer and all other substrates were used at 1 mg/ml. The substrates and buffers used were amylopectin-azure in 0.1 M sodium phosphate buffer, pH 6.2; amylose-azure in 0.1 M sodium phosphate buffer, pH 6.2; blue dextran (Pharmacia Fine Chemicals, Piscataway, NJ) in 0.1 M potassium phosphate buffer, pH 6.2 (Donkersloot and Harr, 1979); carboxymethylcellulose in 0.1 M sodium phosphate buffer, pH 6.2 (Teather and Wood, 1982); casein in 0.1 M citrate-phosphate buffer, pH 5.0; in 0.1 M sodium phosphate buffer 7.0; and in 0.05 M Tris-HCl buffer, pH 9.0 (Durand et al., 1972); 4-methylumbelliferyl (MU)-ß-D-cellobiopyranoside; MU-ß-D-galactopyranoside; MU-α-D-glucoside; MU-ß-D-glucoside; and MU-ß-D-glucuronide in 0.1 M phosphate buffer, pH 6.6; pectin in HEPES buffer, pH 8.0

(Durrands and Cooper, 1988); MU-phosphate in citrate-phosphate buffer, pH 5.0, and 0.05 M Tris-HCl buffer, pH 9.0; sodium polypectate in 0.1 M sodium-citrate buffer, pH 5.0 (Durrands and Cooper, 1988); remazol brilliant blue-xylan (Biely et al., 1985); and MU-ß-D-xyloside in 0.1 M sodium phosphate buffer, pH 6.6. Enzyme samples (10 µl) were added to 2 mm diameter wells that were cut from the agar media with a holepunch. Then the plates were incubated overnight at 35°C. Enzyme action on the substrates was detected by the presence of concentric zones of activity surrounding the agar wells. Depending on the substrate, activity zones were indicated by the formation of clearing zones (amylopectin-azure, amylose-azure, blue dextran, casein, remazol brilliant blue-xylan) or release of the fluorescent 4-methylumbelliferone product which was visualized under long-wave (360 nm) UV-light. Activity zones on the CMC agar medium were enhanced by staining with 0.1% Congo red for 30 minutes at room temperature and destaining with 1 N NaCl for 10 minutes. Activity zones on pectin and sodium polypectate agar media were detected by flooding the gels with 1% cetyltrimethyl ammonium bromide (CTAB) for 5 minutes at room temperature; zones of clearing (enzyme activity) appeared against a whitish (opaque) background of precipitated substrate. Peroxidase activity was detected in 0.1 M phophate buffered (pH 6.8) 1% agar

medium by flooding the gel with 0.01 M o-dianisidine in 3% H_2O_2 and incubating at room temperature until a colored product developed.

Carboxymethylcellulase (CMCase or endo-1,4-ß-glucanase) digest assays also were performed. CMCase activity was measured by using a copper-bicinchoninate reducing sugar test (Waffenschmidt and Jaenicke, 1987) as modified by Fox and Robyt (1991). A copper-bicinchoninate working reagent was prepared daily by mixing two stock solutions in equal volumes. Stock solution A consisted of 2,2'-bicinchoninate dissolved in a solution of 3.2 g $Na_2CO_3 \cdot H_2O$ and 1.2 g $NaHCO_3 \cdot H_2O$ in 45 ml of dH_2O; the volume was adjusted to 50 ml with dH_2O. Stock solution B consisted of 62 mg of $CuSO_4 \cdot 5H_2O$ and 63 mg of L-serine dissolved in 45 ml of dH_2O and adjusted to 50 ml. The substrate solution consisted of 0.5% low-viscosity carboxymethylcellulose (CMC, Fluka Chemical Corp., Ronkonkoma, NY) in sodium acetate buffer (0.05 M, pH 5.0) containing 0.02% sodium azide. The reaction mixture consisted of 0.2 ml of enzyme preparation and 4.8 ml of substrate solution. The reaction mixture was incubated for 20 minutes at 50°C while being mixed at 150 rpm in a reciprocating water bath (Orbit Microprocessor Shaker Bath, Lab-Line Insruments Inc. Melrose Park, IL). Samples (0.3 ml) were removed at 2-minute intervals. Each sample was mixed with an equal volume of copper-bicinchoninate working

reagent and placed into a well of a microtitration plate (Immulon 1, flat bottom, Dynatech Laboratories Inc, Chantilly, VA). Triplicate analyses were performed at each sample time. The plate was covered with a 76 mm x 128 mm strip of transparent tape and then was incubated for 35 minutes at 80°C. Absorbance was measured at 560 nm after the plate had cooled at room temperature for 15 minutes. Absorbance values were subtracted from enzyme and substrate blanks to correct for reducing groups attributed to the digest reagents. A glucose standard (0.1 to 1 μg) was prepared by plotting glucose concentration versus absorbance at 560 nm. The amounts of reducing sugars released from the digests were determined from the standard curve. One unit of enzyme activity was the amount of enzyme liberating 1 uM of reducing sugars in 1 minute.

CMCase and xylanase activities were detected in polyacrylamide gels (PAGs), as described by Schwarz et al. (1987). Details regarding enzyme-activity detection in polyacrylamide gels is presented under the heading "Zymogram Analyses."

Enzyme Preparation for Electrophoresis

Culture fluid (200 ml) from *A. niger* ATCC 9095 was filtered through glass wool to remove most of the mycelia.

The filtrate was further clarified by centrifugation at 10,000 x g for 30 minutes at 4°C. The supernatent was freeze-dried and stored at 4°C. To prepare a sample for analysis, a 200-mg portion of the freeze-dried material was resuspended in 1 ml of 0.02 M Tris-HCl buffer, pH 6.8. Insoluble material was sedimented by centrifugation at 10,000 x g for 5 minutes. The supernatent was desalted in Tris-HCl buffer by using a Bio-Gel P-2 (Bio-Rad laboratories) gel filtration column (1.5 cm x 6 cm). Centrifugation and gel filtration were performed at 4°C. The desalted enzyme sample was stored at -100°C in 5% glycerol. A 400-µl volume of desalted enzyme sample was ultrafiltered by centrifugation (3,000 x g) at room temperature until 200 µl was collected in the ultrafiltrate.

A preparation of a 30-33 kDa *Bacillus subtilis* endoglucanase was recieved as an ammonium sulfate precipitate from Dr. G. Willick (1991). A 200-µl portion of the endoglucanase sample was pelleted by centrifugation at 10,000 x g for 5 minutes. The supernatent was removed and the pellet was resuspended in 200 µl of 0.02 M Tris-HCl buffer, pH 6.8. The protein was reprecipitated with 1 ml of cold acetone (-20°C) and stored overnight at -20°C. The precipitate was pelleted by centrifugation, the supernatent removed, and the residual acetone was evaporated from the pellet by using a stream of ambient air. The pellet was

resuspended in 200 µl of 0.02 M Tris-HCl buffer, pH 6.8. A 100-µl volume of desalted enzyme was ultrafiltered by centrifugation (3,000 x g) at room temperature until 50 µl was collected in the ultrafiltrate.

Electrophoresis

SDS-polyacylamide gel electrophoresis, using a tricine system, was performed as described by Schagger and von Jagow (1987). Gels were prepared from a stock solution that consisted of 48 g acrylamide and 1.5 g N,N-bis-methylene-acrylamide dissolved in 50 ml dH_2O; the final volume was adjusted to 100 ml. The final composition of the acrylamide stock mixture was 49.5% T, and 3% C. T denotes the total percentage concentration of both monomers (acrylamide and bisacrylamide) and C represents the percentage concentration of bisacrylamide relative to the total concentration of both monomers. The gel buffer consisted of 3% SDS in 3.0 M Tris; the pH was adjusted to 8.45 with concentrated HCl. Separating gels (10% T, 3% C) consisted of 6.1 ml acrylamide stock, 4 g of glycerol, 10 ml of gel buffer and 30 ml of dH_2O. The gel mixture was degassed under vacuum for 10 minutes. Polymerization was initiated by the addition of 150 µl of a 10% ammonium sulfate solution and 15 µl of TEMED. The separating gel solution was cast into a vertical

glass plate sandwich assembly (for a 16 cm x 16 cm x 1 mm polyacrylamide slab) until a height of 14 cm was reached. The gel solution was overlaid with a 0.5-cm layer of water. The polyacrylamide was incubated at room temperature for 1 hour to solidify. The final dimensions of the gel were 14 cm x 16 cm x 1 mm. A fifteen-well comb assembly was attached to the top of the glass plate sandwich. The stacking gel consisted of 1 ml acrylamide stock, 3.1 ml gel buffer, and 12.5 ml dH_2O. Polymerization of the stacking gel was initiated by the addition of 50 µl of a 10% ammonium sulfate solution and 5 µl of TEMED. After removing the water layer, the stacking solution was overlaid onto the separation gel until the comb wells were immersed. Dimensions of the stacking gel were 2 cm x 16 cm x 1 mm. Enzyme samples (1-30 µg) in 20-µl volumes were mixed with equal volumes of 2X sample buffer and the mixture was heated at 95°C for 5 minutes before loading the stacking gel. Sample buffer (2X) consisted of 6% SDS and 10% glycerol in 0.12 M Tris-HCl adjusted to pH 6.8 with concentrated HCl. Molecular mass protein markers were prepared as 1 mg/ml stock solutions in 0.06 M Tris-HCl buffer, pH 6.8. The protein markers were mixed (4 µl each) with 20 µl of 2X sample buffer and were heated at 95°C for 5 minutes. Molecular mass protein markers (Boehringer Mannheim, Indianapolis, IN) used were trypsin inhibitor (21.5 kDa),

cytochrome c (12.5 kDa) and aprotinin (6.5 kDa). Four microliters of 0.8% m-cresol purple were added to each 40-μl sample as the tracking dye. Sample buffer was diltued to 1X with dH_2O and loaded into blank sample wells in the stacking gel. Cathode buffer (pH 8.25), which consisted of 0.1 M Tris-HCl, 0.1 M tricine, and 0.1% SDS, was added (400 ml) to the top buffer chamber in the electrophoresis unit (Protean II xi electrophoresis unit, Bio-Rad Laboratories, Bedford, MD). Anode buffer was composed of 0.2 M Tris and the final pH was adjusted to 8.9 with concentrated HCl. Anode buffer (1.8 L) was added to the bottom buffer chamber. Electrophoresis was performed at room temperature. For cooling, 500 ml of an ethylene glycol:water (20:80) solution was added to the cooling core. Electrophoresis (Model 494 power supply, Isco, Inc, Lincoln, NE) was started at 30 V. When the sample entered the stacking gel, the conditions were changed to 150 V until the tracking dye (m-cresol purple) migrated 1 cm from the separating gel bottom.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a glycine system, was performed in polyacrylamide slab gels (16 cm x 16 cm x 1 mm) in the presence of SDS as described by Laemmli (1970). Gels were prepared from an acrylamide stock solution (30% T, 2.6% C) that consisted of 30% (w/v) acrylamide and 0.8% N,N'-Bis-methylene-acrylamide. The separating gel (10% T, 3% C) was composed of 33.3 ml

acrylamide stock, 3 ml glycerol, 1 ml 10% SDS, 500 µl 10% ammonium persulfate, 50 µl TEMED, 25 ml 1.5 M Tris-HCl, pH 8.8, and 31.1 ml dH_2O. The 10% ammonium persulfate solution was prepared daily. CMC (0.1%) was incorporated into the separating gel prior to polymerization. The stacking gel (4% T, 3% C) consisted of 1.3 ml acrylamide stock solution, 100 µl of 10% SDS, 50 µl of 10% ammonium persulfate, 10 µl of TEMED, and 2.5 ml of 0.5 M Tris-HCl (pH 6.8), and 6.1 ml of dH_2O. Separating and stacking gel solutions were prepared by combining all reagents except the 10% ammonium sulfate and TEMED. The mixtures were degassed under vacuum for 15 minutes. Polymerization of the separating gel was initiated by adding 500 µl 10% ammonium persulfate solution and 50 µl TEMED to the separating gel solution (93.5 ml). The gel was cast as described for the tricine SDS-PAGE system. Polymerization of the stacking gel was initiated by adding 50 µl of the 10% ammonium persulfate solution and 10 µl of TEMED to the stacking gel solution (10 ml). The stacking gel was cast as previously described for the tricine SDS-PAGE system. The electrode buffer (pH 8.3), prepared as a 5X solution, contained 72 g glycine, 15 g Tris base, 5 g SDS and dH_2O to 1 liter. The electrode buffer was added to the upper (cathode; 400 ml) and lower (anode; 1800 ml) buffer chambers. Enzyme samples were heated in sample buffer as described for the tricine system. Molecular mass

protein markers were prepared as 1 mg/ml stock solutions in 0.06 M Tris-HCl, pH 6.8. Molecular mass protein standards (4 μl of each protein) were mixed with 20 μl of sample buffer that contained 2% 2-mercaptoethanol (final concentration), and were heated at 95°C for 5 minutes. Molecular mass markers (kDa) used were phosphorylase B (97.4), bovine serum albumin (66), egg albumin (45), carbonic anhydrase (29), and trypsin inhibitor (20). m-Cresol purple was used as the tracking dye and 1X sample buffer was added to each blank sample well in the stacking gel. Electrophoresis was performed at room temperature and the apparatus was cooled as described previously. A current of 16 mA was used during sample migration through the stacking gel and 20 mA during sample migration through the separating gel. Electorphoresis was halted when the tracking dye reached 1 cm from the gel bottom (about 4 hours). Protein bands were detected with a silver-stain kit (Sigma). Molecular mass standard curves were prepared for each electrophoresis run by plotting the log of the molecular mass versus the migration (cm) of the protein markers from the top of the separating gel (Hames and Rickwood, 1981).

Zymogram Analyses

After separation of the enzyme samples by means of SDS-PAGE, CMCase and xylanase activities were detected directly in the separating gels. CMCase activity was detected as described by Schwarz et al. (1987) except that dithiothreitol in 0.1 M succinate buffer was not used to wash the gels. After electrophoresis, the gels were washed for 1 hour with three changes of distilled water, soaked for 10 minutes in 0.05 M sodium acetate buffer (pH 5.0) at room temperature and incubated for 45 minutes at 45°C. The acidic gels were neutralized with Tris-HCl (0.1 M, pH 9.0) for 20 minutes and stained with 0.1% congo red solution for 30 minutes. Pale red hydolysis zones (CMCase zones) emerged against a red background after destaining with 1 M NaCl (about 1 hour). Contrast enhancement of the hydrolysis zones was achieved by soaking the gel in a protein-fixative solution (50% methanol:10% acetic acid:40% dH_2O for 10 minutes). Molecular mass markers, protein profile, and CMCase activity patterns were all detected in the same separating gel by dividing the gel into sections after electrophoresis. After protein and activity pattern development, the gel sections were brought back together so that direct comparisons could be made and molecular mass estimates could be assigned to each zone or band.

Xylanase activity was detected in the separating gel as described with CMCase activity except that 0.1% xylan, instead of CMC, was used as the substrate. Protein pattern and molecular mass markers were detected by staining the gel with 0.1% Serva Blue G (Serva Chemical Co., Westbury, NY) for 1 hour after xylanase activity band development on the same gel without dividing in half.

RESULTS

Ultrafilter Passage Tests

Preliminary experiments using proteins of known molecular mass were conducted to determine if the 10 kDa exclusion membranes would perform as claimed by the manufacturer. Ultrafilter passage tests of ß-galactosidase (540 kDa), bovine serum albumin (66 kDa), trypsin inhibitor (20 kDa), lysozyme (14.4 kDa), cytochrome c (12.5 kDa), and a *Chainia* sp. xylanase (5 kDa) are summarized in Table 1. None of the proteins were detected in the ultrafiltrates, except for cytochrome c and xylanase. Six micrograms, or 1.5% of the original (400 µg) cytochrome c protein, was detected in the ultrafiltrate. Xylanase activity was qualitatively determined by using radial gel diffusion. The protein concentration of the xylanase preparation could not be determined because of the presence of a dark pigment which interfered with the dye-binding protein assay.

Screening for the Presence of Low Molecular Mass Enzymes

Enzyme activities associated with the bacterial and fungal isolates, commercial preparations, and mammalian tissue samples that were examined for small enzymes are

Table 1. Filter passage tests using the Millipore UF MC PTGC ultrafilter units with a 10 kDa exclusion polysulfone membrane.

Solute tested[a]	Molecular mass	Solute in ultrafiltrate[b]
ß-galactosidase	540 kDa	Not detected
Bovine serum albumin	66 kDa	Not detected
Trypsin inhibitor	20 kDa	Not detected
Lysozyme	14.4 kDa	Not detected
Cytochrome c	12.5 kDa	Protein detected
Xylanase	5 kDa	Activity detected

[a]Each protein was filtered at a concentration of 400 µg in 400-µl volumes of 0.05 M Tris-HCl buffer, pH 6.8.

[b]The presence of bovine serum albumin, trypsin inhibitor, and cytochrome C in ultrafiltrates was determined by using a Coommassie Brilliant Blue G-250 dye-binding assay. The presence of ß-galactosidase, lysozyme, and xylanase in ultrafiltrates was determined by radial diffusion assays.

listed in Table 2. A total of 257 samples were screened. For 64 samples, the original 19 substrate profile (Profile A) was used, and 193 samples were screened by using an abbreviated 11 substrate profile (Profile B). Substrate profiles A and B are listed in Table 2. Altogether, 165 bacterial isolates, 79 fungal isolates, 10 commercial preparations, and 3 mammalian tissue samples were examined (Table 2).

4-Methlyumbelliferyl-phosphate at pH 9.0 was the substrate that was most frequently degraded by the bacterial isolates in both profiles A and B (62 isolates). 4-MU-cellobiopyranoside, 4-MU-glucuronide, H_2O_2 (o-dianisidine-positive), and sodium polypectate were not degraded by any of the bacterial isolates in profile A. Amylose-azure, 4-MU-cellobiopyranoside, and H_2O_2 (o-dianisidine-positive) were not degraded by any bacterial isolates in profile B.

Carboxymethylcellulose (degraded by 70 isolates) and RBB-xylan (degraded by 69 isolates) were the most frequent substrates degraded by fungal isolates in both profiles A and B (Table 3). Blue dextran and pectin were not degraded by any fungal isolate in profile A. Amylose-azure, 4-MU-α-D-glucoside, and 4-MU-ß-D-glucuronide were not degraded by any fungal isolate in profile B. The commercial enzyme preparations did not demonstrate activity toward blue dextran or H_2O_2 (o-dianisidine) in profile A and amylose-

Table 2. Summary of enzyme activities detected from bacteria, fungi, commercial enzyme preparations, and mammalian tissue samples.

Substrate[a]	Number of Samples that Degraded Substrates						
	Bacteria		Fungi		CP[b]		MT[c]
Profile[d]	A	B	A	B	A	B	B
Screened[e]	27	138	29	50	8	2	3
Amylopectin-azure	3	-	8	-	8	-	-
Amylose-azure	5	0	2	0	8	0	0
Carboxymethylcellulose	1	15	20	50	8	2	0
Casein, pH 5.0	1	-	3	-	6	-	-
Casein, pH 7.0	1	-	1	-	3	-	-
Casein, pH 9.0	4	-	3	-	8	-	-
MU-cellobiopyranoside	0	0	7	15	8	2	0
Blue Dextran	1	-	0	-	0	-	-
MU-ß-galactoside	7	30	5	23	8	2	2
MU-α-glucoside	3	37	1	0	4	0	1
MU-ß-glucoside	7	26	17	35	8	2	0
MU-ß-glucuronide	0	3	1	0	5	0	0
H_2O_2/o-dianisidine	0	0	1	2	0	0	0
Pectin	2	-	0	-	8	-	-
MU-phosphate, pH 5.0	3	44	3	13	7	0	2
MU-phosphate, pH 9.0	15	47	22	26	7	0	0
Sodium polypectate	0	-	1	-	1	-	-
RBB-Xylan	8	10	19	50	8	2	0
MU-ß-xyloside	2	-	1	-	1	-	-

[a]Enzyme activity on the listed substrates was determined by radial diffusion.

[b]CP=Commercial enzyme preparations.

[c]MT=Mammalian tissue samples.

[d]Profile A included assays for all the enzyme activities listed. Profile B did not include assays for all the enzyme activities listed. Enzyme activities that were not assayed in profile B are indicated by -.

[e]Numbers of bacterial and fungal isolates, commercial preparations, and mammalian tissue samples that were screened for low molecular mass enzymes.

azure, 4-MU-α-D-glucoside, 4-MU-ß-D-glucuronide, H_2O_2 (o-dianisidine), and 4-MU-phosphate at pH 5.0 or pH 9.0 in profile B. Amylose-azure, carboxymethycellulose, 4-MU-ß-D-cellobiopyranoside, 4-MU-ß-D-glucoside, 4-MU-ß-D-glucuronide, H_2O_2 (o-dianisidine), 4-MU-phosphate (pH 9.0), and RBB-xylan were not degraded by the mammalian tissue samples in profile B.

The microorganisms that produced enzymes and commercial enzyme preparations that were ultrafiltrate-positive for CMCase and xylanase activities are listed in Table 3. Six named fungal species, eight unidentified fungal isolates, and two commercial enzyme preparations produced CMCase and xylanase activities that were detected in ultrafiltrates. A 30-33 kDa endoglucanase sample from *B. subtilis* also was ultrafiltrate-positive for CMCase activity (data not shown). A crude xylanase preparation from a *Chainia* sp. was ultrafiltrate-positive for xylanase activity (Table 1).

Zymogram Analyses

Zymogram analysis using tricine buffered SDS-PAGE

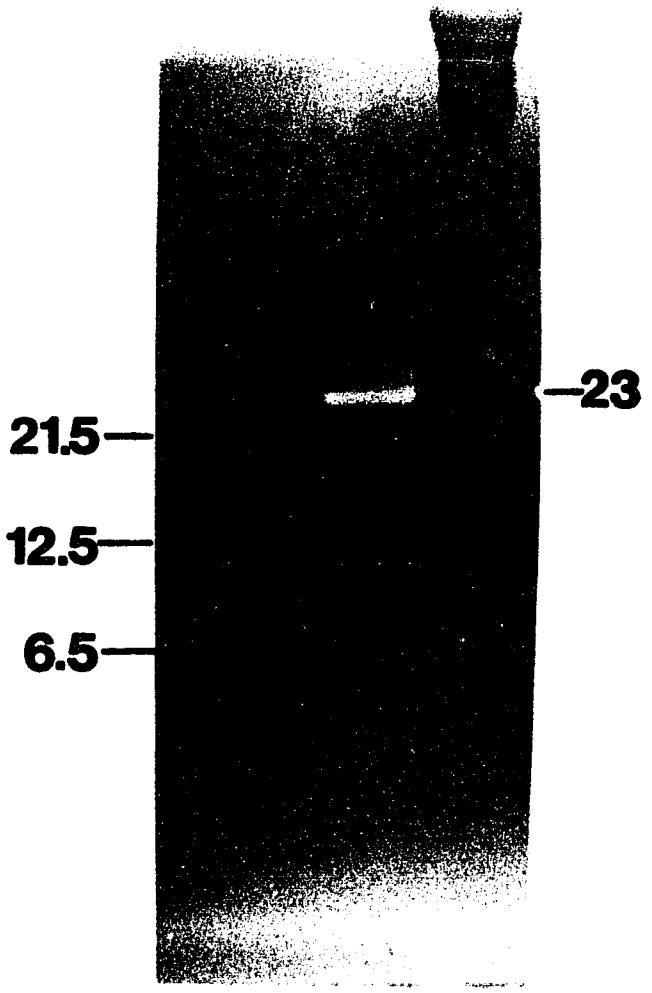
Zymogram (xylanase) analysis of the *A. niger* ATCC 9029 enzyme sample is shown in Figure 1. Two sets of protein molecular mass markers are shown in Figure 1. Cyanogen bromide cleavage fragments of horse myoglobin (Sigma) are

Table 3. Microorganisms and commercial enzyme preparations that were ultrafiltrate (10 kDa exclusion) positive for CMCase and xylanase activities.

Microorganism			
Identified	Unidentified		CP[a]
Aspergillus fumigatus ATCC 46324	SH3	SH42	Fluka
Aspergillus niger ATCC 9095	SH5	SH46	Rohm HE
Diplodia gossypina ATCC 26123	SH13		
Phanerochaete chrysosporium ME446	SH18		
Phlebia tremellosus PRL 2845	SH26		
Trametes versicolor ATCC 12679	SH40		

[a]CP=Commercial enzyme preparation.

Figure 1. Zymogram analysis (xylanase) of an *A. niger* ATCC 9029 enzyme preparation. Before electrophoresis, the enzyme preparation was subjected to ultrafiltration (10 kDa exclusion) and samples of the ultrafiltrate and retentate were examined by zymogram analysis. Samples were denatured in 3% SDS (final concentration) at 60°C for 30 minutes and were subjected to 10% SDS-PAGE using a tricine system. Protein and activity bands were detected in the gel as described in "Materials and Methods". Two sets of protein molecular mass markers are shown in Figure 1 (unmarked lane and lane 1). Cyanogen bromide cleavage fragments of horse myoglobin are shown in the unmarked lane. Trypsin inhibitor (21.5 kDa), cytochrome c (12.5 kDa), and aprotinin (6.5 kDa) are shown in lane 1. The markers in lane 1 were used to estimate molecular mass values of activity bands. Values (kDa) of protein molecular mass markers in lane 1 are on the left. The 6.5 kDa protein molecular mass marker band in lane 1 was lost during photographic reproduction of the master print. The following amounts of enzyme samples were used; lane 2 (ultrafiltrate), 4 μg; lane 3 (retentate), 30 μg.

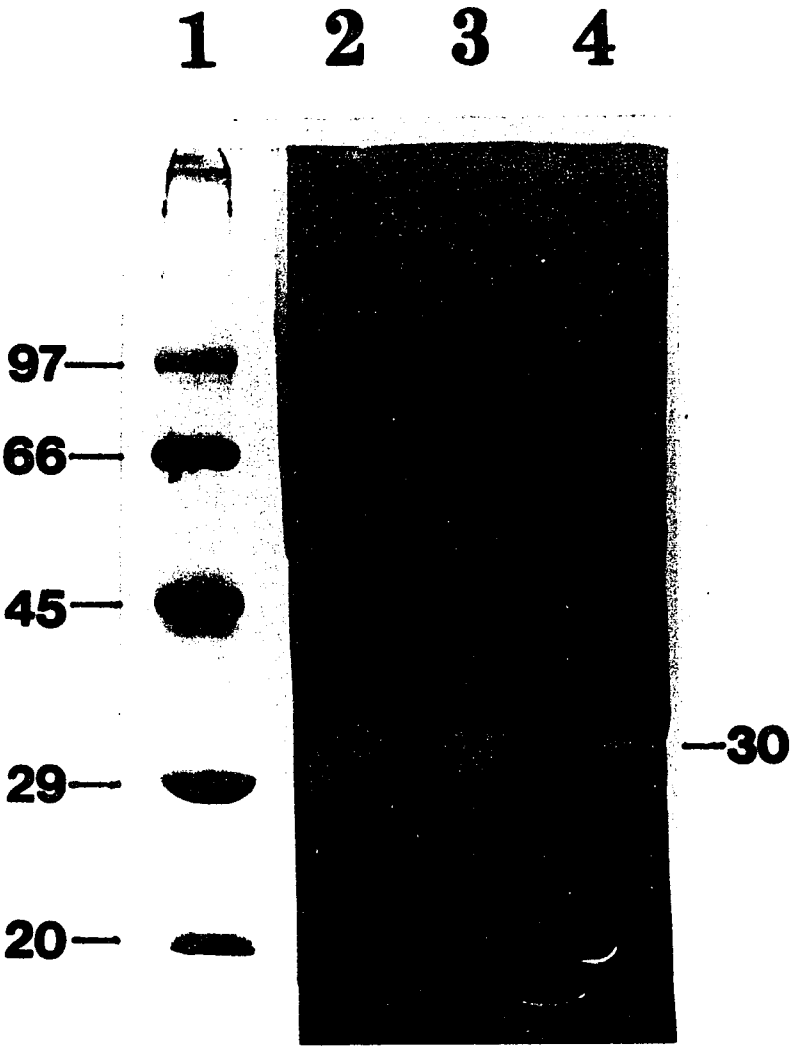

1 2 3
21.5
12.5
6.5
23

shown in the unmarked lane. The 6.5 kDa molecular mass marker in lane 1 was lost during photographic reproduction. The molecular mass markers in lane 1 were used to estimate molecular mass values of activity bands. Values (kDa) of protein molecular mass markers in lane 1 are on the left. The *A. niger* enzyme preparation was subjected to ultrafiltration and samples from the ultrafiltrate (4 μg, lane 2) and retentate (30 μg, lane 3) were examined by zymogram analysis (Figure 1). A strong xylanase activity band at 23 kDa was produced by the ultrafiltrate (lane 2) and a weak band at 23 kDa by the retentate (lane 3). A faint xylanase band was evidient immediately above the 23-kDa band in lane 2. A dark-stained protein band was present in lane 2, above the 23-kDa xylanase band.

Zymogram analysis using glycine SDS-PAGE

A purified 30-33 kDa endoglucanase from *B. subtilis* PAP 115 was subjected to denaturing SDS-PAGE and zymogram (CMCase) analysis (Figure 2) to confirm the molecular mass of the enzyme that passed through the ultrafiltration membrane (10 kDa exclusion). The *B. subtilis* endoglucanase preparation was subjected to ultrafiltration (10 kDa exclusion) and samples from the whole preparation (lane 2), retentate (lane 3), and ultrafiltrate (lane 4) were subjected to zymogram analysis. All samples were denatured with 3% SDS at 95°C for 5 minutes, were subjected to 7.0%

Figure 2. Zymogram (CMCase) analysis of a purified endoglucanase sample from *B. subtilis* PAP115. Before electrophoresis, the endoglucanase preparation was subjected to ultrafiltration (10 kDa exclusion) and samples of whole preparation, ultrafiltrate and retentate were examined by zymogram analysis. The enzyme samples were denatured in 3% SDS (final concentration) at 95°C for 5 minutes and were subjected to 7.0% SDS-PAGE using a glycine buffer. After electrophoresis, the separating gel was divided into two sections consisting of lane 1, and lanes 2-4. Lane 1, containing the molecular mass markers, was stained for protein as described in "Materials and Methods". Values (kDa) of protein molecular mass markers in lane 1 are on the left. Lanes 2-4 contained the endoglucanase samples and were stained for CMCase activity using 0.1% congo red as described in "Materials and Methods". The following amounts of enzyme sample were used; lane 2 (whole sample), 1.5 ug or 1 U; lane 3 (retentate), 1 ug or 0.7 U; lane 4 (ultrafiltrate), undetermined concentration.


1
2
3
4
97
66
45
29
20
30

SDS-PAGE using a glycine-buffered system, and were stained as described in the Figure 2 legend. A 30-kDa CMCase activity band was produced by the whole sample (lane 2), retentate (lane 3), and ultrafiltrate (lane 4). The activity band in lane 4 was the least prominent.

DISCUSSION

Ultrafilter Passage Tests

The first preliminary experiments in a search for low molecular mass enzymes were conducted to determine the ability of ultrafiltration membranes to separate proteins in the desired range size (10 kDa). Passage tests using proteins with known molecular masses were performed to confirm the rated molecular mass exclusion (10 kDa) of the Millipore ultrafiltration membranes and to establish that the *Chainia* sp. xylanase, reportedly 5 kDa in size, could be detected in the ultrafiltrate. Cytochrome c (12.5 kDa) was the only protein with a molecular mass over the rated molecular mass exclusion of the ultrafiltration membrane that was detected in the ultrafiltrate (Table 1). From 400 μg of cytochrome c loaded in the ultrafiltration unit, only 6 μg of protein were detected in the ultrafiltrate. The rejection coefficent for an ultrafiltration membrane is calculated by taking the difference between 1 and the ratio of permeate concentration to feed concentration (Cheryan, 1986). It has been reported that the Millipore PTGC ultrafiltration membrane has a rejection rate of 99% for cytochrome c (Cheryan, 1986) which is consistent with the rejection rate (98.5%) obtained in this study.

The *Chainia* sp. xylanase was originally believed to have a molecular mass of about 5 kDa (Reilly, unpublished results). Futher examination of the enzyme by Reilly and coworkers, however, indicated that the molecular mass may be larger than 5 kDa. Regardless of its "true" molecular mass, xylanase activity from the crude preparation was detected in the ultrafiltrate. Protein concentration in the retentate or ultrafiltrate was not determined because of the presence of a dark brown pigment in the xylanase preparation that interfered with the dye-binding protein assay. Xylanase activity was qualitatively determined by using a radial diffusion method as described in "Materials and Methods". From the results of the preliminary studies, the ultrafiltration procedure should accomplish sufficient separation by molecular mass to achieve its intended purpose.

Screening for the Presence of Low Molecular Mass Enzymes

For the first time, a screening procedure was developed to detect enzymes less than 10 kDa in size. Ultrafilters (10 kDa exclusion) were used to separate sample components larger than 10 kDa in size from the ultrafiltrates. The ultrafiltrates and retentates were then assayed for enzyme activities by using radial gel diffusion in substrate-

incorporated agar media. By using preassembled ultrafiltration units and radial gel diffusion enzyme assays, up to 25 samples were screened simultaneously.

Bacteria and fungi, commercial enzyme preparations, and mammalian tissue samples were screened for the presence of low molecular mass enzymes (Tables 2).

It was not surprising that 4-methylumbelliferyl-phosphate (pH 9.0) was the most frequent substrate hydrolyzed by the bacterial isolates (38%, Table 2) because phosphatase activity has been described from a wide variety of microorganisms (Bayliss et al., 1948; Porschen and Spaulding, 1974; Satta et al., 1979). The lack of a specific enzyme inducer in the enzyme induction broth could have resulted in the failure of some substrates (4-MU-ß-D-cellobiopyranoside, 4-MU-glucuronide, H_2O_2, and polypectate) to be degraded by some of the bacterial isolates. Alternatively, the enzymes needed to hydrolyze the substrates were not released from the cell by the lysis procedure, were inactivated during the lysis procedure, or were uncommon among the bacterial isolates tested. No enzyme activity was detected in the ultrafiltrates of any of the bacterial isolates tested.

Cellulose was used as the sole carbon source in the fungal enzyme induction broth because it has been reported to induce many polysaccharide-degrading enzymes and

glycosidases (Riou, et al., 1991). CMC (degraded by 70 isolates) and RBB-xylan (degraded by 69 isolates) were the most frequent substrates degraded by the fungal isolates (Table 2). Reports that cellulase and xylanase activities are often associated in fungal extracellular culture fluid have been described (Ball and McCarthy, 1989; Wong et al., 1988). Six identified fungal cultures and eight unidentified isolates possessed CMCase and xylanase activities that were detected in ultrafiltrates (Table 3).

Most of the commercial enzyme preparations were from Rohm Tech and are used for baking applications. Two preparations (Fluka and Rohm Tech HE) possessed CMCase and xylanase activities that passed through an ultrafilter with a 10 kDa exclusion (Table 3). The Fluka enzyme source was described as a xylanase preparation from *Trichoderma viride*. The Rohm Tech HE enzyme source was a hemicellulase preparation formulated to reduce baking dough viscosity (Rohm Tech Inc, 1988).

Samples of bovine, chicken, and swine liver tissues were examined for small enzymes because it was reported that an 11.5 kDa acid phosphatase was isolated from bovine liver homogenate (Millipore Technical Bulletin, 1989). Although acid phosphatase activity was present in the retentates of bovine and swine livers (Table 2), no activity was detected in the ultrafiltrate.

A purified endoglucanase (30-33 kDa) sample from *B. subtilis* PAP 115 was tested for its ability to pass through an ultrafilter (10 kDa exclusion). In this study, it was ultrafiltrate-positive for CMCase activity.

Theoretically, only enzymes with a "true" molecular mass below 10 kDa should pass through a 10-kDa exclusion membrane. In this study, a xylanase (23 kDa) from *A. niger* ATCC 9029 and an endoglucanase (30 kDa) from *B. subtilis* PAP 115 passed through 10-kDa exclusion filters. In addition, tests with globular proteins of known molecular mass demonstrated that the size exclusion claimed by the manufacturer was valid. Other investigators have described a cellulase and xylanases that possessed the ability to penetrate ultrafiltration membranes with a 10 kDa exclusion, even though molecular masses of the enzymes were estimated to be between 20 to 29 kDa (Bhat et al., 1988; Grabski and Jeffries, 1991; Wong et al., 1980). The ability of an enzyme with a molecular mass of 20 kDa or greater to penetrate a 10-kDa exclusion membrane could be attributed to an elongated, tapered, or compact molecular shape that enables passage through pores smaller than its molecular mass would indicate if the enzyme were a truly globular protein.

Zymogram Analyses

Zymogram analysis using tricine buffered SDS-PAGE

A crude extracellular enzyme preparation from *A. niger* ATCC 9029 was subjected to denaturing SDS-PAGE and zymogram (xylanase) analysis to determine the molecular mass of the xylanase component(s) that passed through an ultrafiltration membrane (10 kDa exclusion). A tricine-buffered system for SDS-PAGE analysis was used to achieve optimal resolution within the 1 to 20 kDa range (Schagger and von Jagow, 1987). Information on the xylanase composition produced by *A. niger* ATCC 9029 has not been reported; however, xylanases from other *Aspergillus* spp. range from 11 to 50 kDa in size (Wong et al., 1988). Ultrafiltration of the *A. niger* ATCC 9029 enzyme preparation resulted in the passage of at least one xylanase into the ultrafiltrate (Figure 1). By zymogram analysis, however, the xylanase was assigned a molecular mass of 23 kDa (Figure 1, lane 2), which was greater than the reported exclusion point of the ultrafilter membrane (10 kDa). Grabski and Jeffries (1991) and Wong et al. (1986) reported that xylanases from *Streptomyces roseiscleroticus* and *Trichoderma harzianum* E58 passed through polysulfone ultrafiltration membranes with an exclusion of 10 kDa. The molecular masses of the xylanases were later determined to be 20 to 29 kDa in size by denaturing SDS-PAGE. They did

not determine if the ability of the xylanases to pass through the membranes was because they possessed a compact molecular structure or because the pores in the ultrafilter were not uniform in diameter. The ability of the *A. niger* ATCC 9029 xylanase to penetrate the membrane in this study may have a basis similar to what was described by Grabski and Jeffries (1991) and Wong et al. (1986).

Zymogram analysis using glycine buffered SDS-PAGE

A purified 30 to 33 kDa endoglucanase from *B. subtilis* PAP115 was subjected to denaturing SDS-PAGE and zymogram (CMCase) analysis to confirm the molecular mass of the enzyme that passed through the ultrafilter membrane (10 kDa exclusion). The molecular mass estimate of the endoglucanase (30 kDa) using zymogram analysis (Figure 2, lanes 2-4) was consistent with the reported molecular mass of the enzyme. The ability of the *B. subtilis* endoglucanase to pass through an ultrafilter with a 10-kDa exclusion may also be attributed to its compact molecular shape.

The results of my study and those of other investigations (Bhat et al., 1988; Grabski and Jeffries, 1991; Wong et al., 1980) indicate that enzymes exist that do not conform to the ususal filtration parameters. Although beyond the scope of this study, the unique characteristics of these enzymes should be elucidated. Unusual protein conformation, such as described in this dissertation, may be

more common than previously thought. One question that should be answered is why this unusual property of passage of a 20 kDa protein through a 10-kDa exclusion membrane seems unique to glucanases.

PART II: EVALUATION OF A ZYMOGRAM METHOD FOR MICROBIAL ENDOGLUCANASES

INTRODUCTION

Cellulose is an abundant renewable resource, and its bioconversion has many biotechnological applications. The characterization of cellulose-degrading enzymes, however, has been hampered by the complexity of the cellulase system (Enari and Niku-Paavola, 1987). The cellulase system from *Trichoderma reesei* is comprised of endoglucanases (EC 3.2.1.3; 1,4-ß-D-glucan-4-glucanohydrolase), cellobiohydrolases (EC 3.2.1.9; 1,4-ß-D-glucan cellobiohydrolase), and ß-glucosidases (EC 3.2.1.21; ß-D-glucoside glucohydrolase) (Wood, 1992). The endoglucanases are believed to exist as multiple molecular forms or as heterogeneous enzyme complexes (Labudova and Farkas, 1983; Sprey and Lambert, 1983). Two endoglucanases (endoglucanase I, 55 kDa and endoglucanase II, 50 kDa) from *T. reesei* have been cloned and sequenced (Pentilla et al., 1986; Saloheimo, 1988), but many more endoglucanases have been described (Messner et al., 1988; Sprey and Uelker, 1992). The numbers of *T. reesei* endoglucanases reported in the literature (reviewed by Enari and Niku-Paavola, 1987) often differ. A simple method for identifying the spectrum of endoglucanase molecular forms would facilitate their subsequent isolation, purification, and characterization.

Antibodies have been used to detect endoglucanase

species after SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and protein blotting (Luderer et al., 1991; Messner et al., 1988). Molecular mass values, however, could be assigned only to the protein bands reacting specificlly with antibodies. Enzyme activity could not be directly assayed. Zymogram methods also have been used to identify endoglucanase components from complex protein mixtures (Biely and Markovic, 1988). When nondenaturing gels are used in zymogram methods, enzyme activity is retained (Gabriel and Gersten, 1992). Nondenaturing zymogram systems, however, may not sufficiently resolve enzyme complexes (Sprey and Lambert, 1983), and molecular mass estimates (often used to distinguish between endoglucanases) cannot be assigned to the activity bands. Zymogram protocols using denaturing SDS-PAGE may eliminate problems sometimes associated with nondenaturing zymogram systems. Heat treatment in the presence of SDS may effectively solublize endoglucanase aggregates into individual components that can then be separated by SDS-PAGE (Lamed et al., 1983). The separated enzymes that remain active are identified according to protocols previously described for endoglucanase renaturation and detection in polyacrylamide gels (Beguin, 1983; Schwarz et al., 1987). Molecular mass estimates can be assigned to each activity band. Several investigators have used denaturing zymogram methods to

identify endoglucanase [Carboxymethylcellulase; (CMCase)] species from the thermophilic anaerobe *Clostridium thermocellum* (Beguin, 1983; Lamed et al., 1983; Schwarz et al., 1987). Schwarz et al. (1987) improved detection sensitivity of the method by incorporating the substrate (CMC) directly into the separating gel instead of using a substrate agar replica overlay (Beguin, 1983). The Schwarz et al. (1987) *in situ* zymogram technique was a simple method of characterizing *C. thermocellum* endoglucanase components, but further evaluation of its reliability and compatibility with cellulase sytems from other sources is needed. Cote et al. (1991) recently demonstrated that enzyme extracts from a *Trichoderma* sp. and from other fungi renatured after denaturing SDS-PAGE and that enzyme activity (lichenase or 1,3 ß-glucanase) was detected in substrate-containing gels.

In the present study, endoglucanase components from a crude *T. reesei* cellulase preparation were identified by using the Schwarz et al. (1987) *in situ* zymogram method. Recorded molecular mass estimates were compared with previously reported values. Endoglucanase samples from *Bacillus subtilis* PAP 115, *Myrothecium verrucaria* ATCC 9095, and two commercial cellulase preparations also were examined by zymogram analysis. Potential usefulness of the zymogram technique is discussed.

LITERATURE REVIEW

Cellulose Bioconversion

Plant biomass in the form of cellulose, hemicellulose, and lignin is an abundant renewable resource and its conversion into soluble products has many biotechnological applications (Bisaria, 1981). Most research on biomass conversion has focused on cellulose degradation for the production of glucose (Persson et al., 1991). Glucose can be metabolized by microorganisms during industrial fermentation processes to produce amino acids (Hirose, 1979), antibiotics (Perlman, 1979), ethanol (Bisaria, 1981), enzymes (Aunstrup et al., 1979), and other useful compounds (Bisaria, 1981).

The cellulose molecule is an unbranched homopolymer containing glucose units linked via 1,4-ß-D-glucosidic bonds. Cellobiose is the repeating disaccharide in the celluose chain. Cellulose microfibrils are insoluble aggregates of polyglucose chains that possess a high capacity to form interchain hydrogen bonds (Bohinski, 1983). Native cellulose usually consists of amorphous and crystalline microfibrillar regions. Amorphous microfibrillar regions are loosely hydrogen-bonded, readily hydrated, and are easily accessible to chemical hydrolysis

or enzymatic degradation. Crystalline microfibrillar areas, however, are comprised of highly ordered cellulose chains that result in powerful intermolecular hydrogen bonding. Consequently, crystalline regions are extremely resistant to chemical or enzymatic treatments because the individual celluose chains are inaccessable (Bisaria, 1981).

Cellulose degradation can occur by acid hydrolysis or enzymatic catalysis. Acid-hydrolysis processes are continually being improved, however, current major drawbacks include equipment corrosion and the generation of product impurities.

Successful utilization of cellulose as a renewable resource may depend on the development of efficient enzymatic bioconversion methods. The advantages of using enzymes rather than acids include the avoidance of corrosive chemicals, decreased generation of undesirable side products, and the production of unique cellodextrins by specific enzymatic reactions (Leathram and Himmel, 1991). But before enzymes can be used appropriately and economically in cellulose bioconversion processes, an understanding of their nature and role in cellulose degradation is required.

Cellulose-degrading-enzymes (cellulases) are produced by a wide variety of bacteria (reviewed by Robson and Chambliss, 1989) and fungi (reviewed by Persson et al.,

1991). Most cellulolytic bacteria, such as *Bacillus subtilis*, cannot significantly hydrolyze crystalline cellulose forms; however, they usually can degrade amorphous substrates to varying degrees. The thermophilic anaerobe, *Clostridium thermocellum*, is one of only a relatively few bacteria capable of extensively hydrolyzing crystalline cellulose substrates (Robson and Chambliss, 1989). Some aerobic fungi, such as certain species of *Trichoderma* (Perssom et al., 1991) and *Myrothecium* (Selby and Maitland, 1964), secrete large amounts of cellulase into culture media and can solubilize crystalline cellulose. As will be described later, the cellulase system of *Trichoderma reesei* consists of multiple enzyme components that act independently and in a cooperative fashion to extensively degrade crystalline cellulose forms.

Fungal Cellulases

Many aerobic fungal genera, including *Aspergillus*, *Penicillium*, *Phanerochaete*, and *Myrothecium*, can degrade cellulose; however, much of the cellulase research on fungal cellulases has focused on *Trichoderma* spp. (Mandels and Andreotti, 1978). Contrary to most cellulolytic fungi, *Trichoderma* spp. secrete a complete cellulase system that consists of components that hydrolyze crystalline cellulose

(Ryu and Mandels, 1980). *Trichoderma* spp., such as *T. koningii*, *T. lignorum*, *T. reesei*, and *T. viride*, produce an active and well-balanced cellulase complement. The development of hyper-cellulase-producing and catabolite-repression-resistant mutants of *T. reesei* greatly facilitated research on *Trichoderma* cellulases. For example, *T. reesei* QM 9414 is a mutant that produces twice as much cellulase as the wild type *T. reesei* QM 6a (Persson et al., 1991).

The cellulase system secreted by *T. reesei* consists mainly of cellobiohydrolases (EC 3.2.1.9; 1,4-ß-D-glucan cellobiohydrolase), endoglucanases (EC 3.2.1.3; 1,4-ß-D-glucan-4-glucanohydrolase), and ß-glucosidases [EC 3.2.1.21; ß-D-glucoside glucohydrolase (reviewed by Ryu and Mandels, 1980 and Wood, 1992)]. The cellobiohydrolases are often referred to as exoglucanases because they can degrade cellulose by removing cellobiose units from the nonreducing end of the chain. Amorphous cellulose, soluble cello-oligosaccharides, Avicel (Microcrystalline cellulose), and to a lesser extent, cotton, are hydrolyzed by cellobiohydrolases. But soluble cellulose derivatives, such as carboxymethylcellulose (CMC), are not significantly degraded. The endoglucanases, however, can act on CMC as well as amorphous cellulose forms and soluble cello-oligosaccharides. It is generally accepted that

endoglucanases act on substrates in a random fashion, breaking internal glucosidic bonds of the cellulose chain. Purified endoglucanases have little apparent capacity to hydrolyze crystalline cellulose. Glucose and cellodextrins are the main products of endoglucanase action. The ß-glucosidases can convert cellobiose into glucose, but native cellulose is not degraded. Although ß-glucosidases are required for complete degradation of crystalline cellulose, they are usually not considered true cellulases.

Cellulases are believed to exist in multiple molecular forms that differ in mass, ionic properties, or activities toward specific cellulosic substrates (reviewed by Enari and Niku-Paavola, 1987). Raw native cellulose is intertwined with varying amounts of lignin and hemicellulose, depending on the source of the cellulose (Lipinsky, 1978). In has been suggested that a battery of secreted cellulases, that slightly differ in functional properties, may be required for efficient and effective solubilization of natural cellulose forms found in the environment (Lamed et al., 1983).

The origins of cellulase multiplicity have been attributed to the formation of distinct gene products (Pentilla et al., 1986; Saloheimo et al., 1986), post-translational modifications (Gum and Brown, 1977; Stahlberg et al., 1988), and protein-protein (Dominguez et al., 1992;

Sprey and Lambert, 1985) or protein-carbohydrate (Luderer et al., 1988) interactions. Two cellobiohydrolases [CBH I, 67 kDa (Shoemaker et al., 1983); and CBH II, 56 kDa (Teeri et al., 1987)], and two endoglucanases [EG I, 55 kDa (Pentilla et al., 1986); and EG II, 50 kDa (Saloheimo et al., 1986)] have been cloned and sequenced to date; however, many more cellulases have been identified (Messner et al., 1988). The endoglucanases secreted by *T. reesei* are extremely heterogeneous (Wood, 1992). In addition to the cloned cellulases, low molecular mass endoglucanases, ranging between 20 and 27 kDa in size, have consistently been isolated from culture media (Beldman et al., 1985; Hakansson et al., 1978; Sprey and Uelker, 1992; Uelker and Sprey, 1990). These low molecular mass endoglucanases seem not to be glycosylated, which is in contrast to the cloned endoglucanases (Pentilla et al., 1986; Saloheimo et al., 1986).

Some endoglucanases exist as heterogeneous protein complexes (Sprey and Lambert, 1983) or as large molecular mass aggregates consisting of smaller sized endoglucanase components (Dominguez et ai., 1992; Messner et al., 1988). Sprey and Lambert, (1983) isolated an enzyme aggregate from *T. reesei* that could be dissociated into an endoglucanase, a xylanase, and a ß-glucosidase after treating the complex with a mild detergent. The enzyme complex had previously

been determined to be homogeneous by using simple electrophoresis, gel filtration, and isoelectric focusing. Dominguez et al. (1992) recently demonstrated that endoglucanase I (55 kDa) can spontaneously associate into large molecular mass complexes during gel filtration if the pH of the elution buffer is maintained between 5.5 and 7.0. Using polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE), Messner et al. (1988) identified a 118 kDa protein component that reacted with antibodies prepared against endoglucanase I. The 118 kDa protein could be dissociated into 55 kDa by treating it with chloroform and methanol. Messner et al. (1988) concluded that the 118 kDa component was an SDS-stable dimer of endoglucanase I.

Partial proteolysis of noncatalytic enzyme regions has also been implicated as a mechanism for endoglucanase diversity in *T. reesei* (Luderer et al., 1991; Stahlberg et al., 1988). Stahlberg et al. (1988) isolated a 38 kDa protein from *T. reesei* culture fluid that was active on CMC. The 38-kDa CMCase was identical to endoglucanase II (50 kDa), except that it lacked the first 61 N terminal amino acids. In a separate study, endoglucanase II was cleaved into 45 kDa components after prolonged exposure to papain treatment (Luderer et al., 1988). Enzyme activities of the proteolytic fragments were not determined. Proteolytic activity has also been attributed to be a cause of the

extreme heterogeneity of endoglucanase I exhibited in some commercial cellulase preparations (Kubicek-Pranz et al., 1991).

Several research groups have claimed that differential glycosylation accounts for many of the cellulase forms secreted by *T. reesei* (Gum and Brown, 1977; Gritzali and Brown, 1979). But Enari and Niku-Paavola (1987) suggested that the sugar content attributable to glycosylation is too low, even at the maximum reported amounts (15%-21%), to account for the differences in endoglucanase molecular masses (20 to 55 kDa) reported in the literature.

Because many cellulases can adsorb to cellulose particles, Luderer et al. (1991) hypothesized that endoglucanase multiplicity could be caused by noncovalent binding of hydrolysis products. Regardless of whether cellulase heterogeneity is determined transcriptionally, post-translationally, post-secretionally, or by a combination of the mechanisms, each enzyme group seems to have a specific role in cellulose hydrolysis.

The various groups of cellulases from *T. reesei* work in a synergistic fashion to solubilize crystalline cellulose (reviewed by Wood, 1992). Synergism between cellulase components occurs when the action of two or more enzymes acting cooperatively is greater than the sum of their individual actions. At least three types of cooperative

action between cellulases have been described in *Trichoderma*. Purified cellobiohydrolases, endoglucanases, or ß-glucosidases are unable alone to extensively hydrolyze crystalline cellulose forms. But in combinations of cellobiohydrolases and endoglucanases, or cellibiohydrolase I and II, crylalline cellulose forms are significantly hydrolyzed. A third form of synergism between cellobiohydrolases, endoglucanases, and ß-glucosidases has been described. It has been postulated that the third type of synergism results in the elimination of end-product inhibition of cellobiose on cellobiohydrolase activity. The basis for the first two types of enzyme-cooperative-action, between endoglucanases and cellobiohydrolases, or cellobiohydrolase I and cellobiohydrolase II, is not well understood. It seems, however, that synergism involving crystalline cellulose is the most pronounced, and that involving amorphous or soluble substrate forms is negligible or nonexistent.

A mechanism for enzymatic cellulose hydrolysis was first proposed by Dr. Elwyn Reese and coworkers (1950). The C_1-C_x hypothesis, as it was termed, distinguished between complete and incomplete cellulase systems. As described, the C_1 hydrolytic factor would convert crystalline cellulose into a "reactive" or easily degradable form. Subsequently, the modified cellulose form could be degraded by the C_x

enzymes. The "x" in C_x reflected enzyme heterogeneity. According to Reese et al. (1950), microorganisms with complete cellulases (C_1-C_x) could degrade crystalline cellulose, whereas microbes that possess incomplete systems (C_x) could only hydrolyze amorphous cellulose forms. Although this model was an oversimplification, it remained an inspiration for the development of more refined cellulose degradation mechanisms (Enari and Niku-Paavola, 1987; Ryu and Mandels, 1980).

A recent model for cellulose degradation has been proposed by Enari and Niku-Paavola (1987). They theorized that crystalline cellulose degradation is initiated by the cooperative action of cellobiohydrolases which release cellodextrins and cellobiose. The products liberated by cellobiohydrolase action are subsequently converted to glucose by endoglucanases and ß-glucosidases. All aerobic fungi that possess complete cellulolytic systems (cellobiohydrolases, endoglucanases, and ß-glucosidases) are believed to degrade cellulose by a similar mechanism (Wood, 1992).

Myrothecium verrucaria is an aerobic celluloytic fungus that has been overshadowed by strong interest in the cellulases of *T. reesei* research; however, *M. verrucaria* has acquired some historical significance. *M. verrucaria* (formally known as *Metarrhizium glutinosum*) was isolated in

1943 from rotting baled cotton stored in Washington, DC (White and Downlng, 1946). Fungal decomposition of military fabrics during WW II prompted the development of preventive measures and the subsequent distribution of *M. verrucaria* cultures to research institutions. *M. verrucaria* was one of the first microorganisms used to study fungal degradation of cellulose. Selby and Maitland (1964) fractionated spent culture fluid from *M. verrucaria* into three cellulase components with molecular masses of 55, 30, and 5.3 kDa, respectively. Studies of the *M. verrucaria* cellulolytic system were discontinued, however, after the isolation of *Trichoderma* strains that produced cellulases which were more effective in hydrolyzing native cellulose (Mandels and Reese, 1964).

Knowledge of aerobic fungal cellulases is extensive, but, research on anaerobic cellulolytic fungi has only recently been initiated. Anaerobic phycomycete fungi, such as *Neocallimastix frontalis* and *Piromonas communis*, are believed to have a role in ruminant fiber digestion; most strains possess cellulolytic activity (Bauchop. 1981; Orpin, 1977; Orpin, 1981). Cellulases from anaerobic fungi seem to be cloesly associated with the hyphae, contrary to aerobic fungal cellulases (Bauchop, 1981). Other properties not found in aerobic cellulolytic fungi can be attributed to the anaerobic lifestyle of the phycomycetes (Bauchop, 1981).

Although little is known about the nature of anaerobic fungal cellulases, the study of anaerobic bacterial cellulolytics has progressed rapidly.

Bacterial Cellulases

Anaerobic cellulose-degrading-bacteria have conventionally been isolated from ruminants and are involved in fiber digestion and fermentation (Hespell, 1988). Interest in anaerobic bacterial cellulase systems is attributed to the high specific activites achieved with some strains. In addition, prokaryotes are more amenable than fungi to genetic manipulation (Robson and Chambliss, 1989).

Members of the anaerobic bacterial genera *Bacteroides*, *Clostridium*, and *Ruminococcus*, seem to degrade cellulose by a process that is different than that described for aerobic fungal systems (reviewed by Robson and Chambliss, 1989). Cellulases from anaerobic bacteria are initially bound to the cell surface and may mediate contact between the cell and cellulose fibers. During later stages of cellulose hydrolysis, the cellulases are released into the culture medium and attach to the substrate. It was hypothesized that cell-surface-bound cellulases could ensure the release of hydrolysis products in close proximity of the microorganism, thus facilitating uptake.

The cellulase complex from *C. thermocellum* has been well characterized because of the organism's biotechnological importance. The direct bioconversion of cellulose to ethanol and the ability of *C. thermocellum* to grow at a high temperature (69°C) favor *C. thermocellum* for industrial applications (Wiegel, 1988). The cellulases produced by *C. thermocellum* consist of a cellulose-binding, multienzyme-containing protein complex termed the cellulosome (Bayer et al., 1985). By using cytochemical methods and immunoelectron microscopy, ultrastructural distribution of the cellulosome on the cell surface was examined (Bayer and Lamed, 1986). When *C. thermocellum* was cultivated in cellobiose-containing medium, the cellulosome complexes were compacted into polycellulosomal proturbances on the outer cell surface. When the microorganism was cultivated on a cellulose-based medium, however, the polycellulosomal proturbances attached to the substrate and formed a fibrous network that intermixed with the individual cellulosome complexes. The fibrous network of unknown composition seemed to create a "contact corridor" between the cell and the substrate. Bayer and Lamed (1986) postulated that the corridor may mediate the transfer of soluble cellulose hydrolysis products to the cell. Cellulosomes were released from the cell-wall proturbances during corridor formation and were found free in the culture

medium as well as bound to the cellulose fibers. Cellulolysis occurred at the cellulosome-substrate contact points.

The cellulosome complex was isolated by Lamed et al. (1983) by using chromatographic techniques and was determined to be homogeneous by simple electrophoresis. The complex exhibited a molecular mass of 2.1 million MDa and could not be dissociated using urea treatments. Denaturing SDS-PAGE analysis, however, resolved the aggregate into fourteen polypeptide bands. A 2.1 kDa glycoprotein was the only antigenically active component, and eight of the fourteen bands possessed CMCase activity as revealed by means of zymogram analyses. Recent studies by Mayer et al. (1987) and Kohring et al. (1990), have indicated that the cellulosome polypeptide composition varies among different *C. thermocellum* strains. Electron microscopic examination of cellulosomal particles revealed that they contain rows of polypeptide subunits that were equidistantly spaced. Mayer et al. (1987) therorized that the polypeptide subunits in the cellulosome were cellulase components that took part in a multicutting event after cellulosomal adherence to cellulose. Multiple hydrolytic action by the equidistantly spaced cellulase units could lead to the release of soluble cello-oligosaccharides that are eventually degraded to cellobiose and glucose (Mayer et al., 1987). Fifteen

endoglucanases and 2 ß-glucosidases have been cloned from *C. thermocellum*, and there are indications that more cellulase genes exist (Hazelwood, 1988). Because no cellobiohydrolases have been characterized from *C. thermocellum* thus far (Hazelwood, 1988), the model proposed by Mayer et al. (1987) would account for the complete degradation of cellulose involving only endoglucanases and ß-glucosidases. Some aerobic bacteria also lack cellobiohydrolases; however, they usually cannot significantly degrade crystalline cellulose forms (Robson and Chambliss, 1989).

Bacillus spp. are aerobic and facultatively anaerobic, Gram positive, sporeforming bacteria that can secrete a plethora of enzymes such as amylases, proteases, and glucanases (Priest, 1977). But cellulolytic *Bacillus* strains seem to lack cellobiohydrolase activity, and consequently cannot degrade crystalline cellulose (reviewed by Robson and Chambliss, 1989). Interest in *Bacillus* cellulases is attributed to the well-characterized genome of some species which facilitate genetic manipulation. In addition, the endoglucanses are easy to purify because the enzymes are secreted into the culture medium. The cellulolytic species include *B. cereus*, *B. licheniformis*, *B. polymyxa*, *B. subtilis*, and some alkalophilic strains.

Endoglucanase multiplicity has been reported in

Bacillus spp. and was attributed to the presence of distinct gene products (Fukamori et al, 1986) and proteolytic processing (Lo et al., 1988). Three endoglucanase genes have been cloned and sequenced from the same alkalophilic strain (Fukamori et al., 1986). The endoglucanse heterogeneity observed from *B. subtilis* PAP115, however, seems to result from proteolytic processing of nonessential enzyme regions (Lo et al., 1988). The endoglucanase from *B. subtilis* PAP115 was secreted into the culture medium as a 52.2-kDa proenzyme and was progressively cleaved into a 32-kDa component. Using SDS-PAGE and western blotting, endoglucanase cleavage products were identified with antibodies prepared against a 35.8-Kda active enzyme species isolated from *B. subtilis* PAP115 culture medium. The 35.8-kDa component was identical to the 52.2-kDa proenzyme; however, it lacked a 163-amino acid sequence at the carboxyl terminus. Endoglucanase activities of the individual proenzyme cleavage fragments were not determined.

The last group of cellulolytic bacteria to be discussed include members of the actinomycetes. The actinomycetes consist of aerobic and facultatively anaerobic microorganisms that possess properties similar to the aerobic cellulolytic fungi. These bacteria exhibit fungal-like mycelial growth. Many species are capable of degrading complex polysaccharides, such as cellulose, chitin, lignin,

and peptidoglycan (Stanier, 1988). Well characterized cellulolytic actinomycetes belong to the genera *Cellulomonas*, *Streptomyces*, and *Thermomonospora* (reviewed by Robson and Chambliss, 1989). Cellulases produced by these actinomycetes consist of multicomponent enzyme systems which include cellobiohydrolases, endoglucanses, and ß-glucosidases. The involvement of each enzyme group during cellulose hydrolysis is similar to the aerobic fungal cellulase system and further discussion would be redundant.

Identification and Fractionation Methods Used for Microbial Cellulases

Substrates used to measure cellulase activity

Because of the complex heterogeneous nature of microbial cellulases and the numerous cellulose substrates available (Bisaria, 1980), a variety of methods have been used to measure enzyme activity (Wood and Bhat, 1988). Many of the cellulase forms share overlapping specificities; however, enzyme activity has been catagorized into four main groups: total or complete cellulase, cellobiohydrolase, endoglucanase, and ß-glucosidase activities (Wood and Bhat, 1988).

Complete cellulase activity can be measured by using crystalline cellulose substrates, such as Avicel, cotton

fiber, filter paper, or Solka Floc. Enzyme assays using cotton fiber as a substrate involve measuring fiber weight loss, tensile strength decrease, unhydrolyzed residual cotton, or reducing sugars released. Assays using Avicel, filter paper, or Solka Floc, involve estimating reducing sugars liberated after hydrolysis. Avicel is prepared from cellulose by an acid-blending treatment that aggregates microcrystals of short chain lengths (DP @200; Wood, 1988). Solka Floc is a commercially available acetic acid-treated cellulose that may contain up to 17% xylan and has a degree of polymerization of about 2,000-3,000. Filter paper, which is considered one of the best substrates for measuring complete cellulase activity is recommended by the Commission on Biotechnology (IUPAC; cited by Wood, 1988). The assay is based on estimating the amount of glucose released from a 1 cm x 6 cm strip of Whatman No. 1 filter paper in an enzyme digest. Complete cellulase activity also can be measured by detecting dyed-soluble-oligosaccharides liberated after the hydrolysis of dyed-cellulose or dyed-Avicel substrates. There is no specifc substrate to measure cellobiohydrolase activity, consequently, the enzyme must be purified to prove its existence. Avicel is often used in assays to determine the activty of purified cellobiohydrolases. Because of its short chain length (DP @200), Avicel is readily susceptible to exo-acting (cellobiohydrolases) enzymes.

Cellobiohydrolases generally exhibit negligible activity toward CMC and are inhibited by cellobiose (van Tilbeurgh and Claeyssens, 1985). An assay to selectively determine cellobiohydrolases in crude cellulase preparations was developed by Deshpande et al. (1984); however, the procedure was time consuming and depended on measuring both cellobiohydrolase activity and endoglucanase activity separately.

Endo-acting 1,4-ß-D-glucanase activity has been measured by using soluble cellulose derivatives, such as CMC or hydroxyethlycellulose (HEC; Wood and Bhat, 1988). CMC is the most common endoglucanase substrate and its hydrolysis is measured by detecting the release of reducing sugars or a decrease in viscosity of a digest solution.

ß-Glucosidase activity can be measured by determining the amount of glucose or chromophoric aglycones liberated after hydrolysis of cellobiose or synthetic aryl-ß-D-glucosides (Wood and Bhat, 1988). ß-Glucosidase does not attack CMC and is inhibited by gluconolactone (van Tilbeurgh and Claeyssens, 1984).

Chromatographic methods used for the fractionation of microbial cellulases

Characterization of cellulose-degrading enzymes has been hampered by their complex nature (Enari and Niku-Paavola, 1987). The cellulase system from *T. reesei*

consists of cellobiohydrolases, endoglucanses, and ß-glucosidases. The endoglucanases are believed to exist as multiple molecular forms or as heterogeneous enzyme complexes. Endoglucanse heterogeneity was discussed in more detail under "Fungal Cellulases". Endoglucanase multimers or hetergeneous aggregates often were not dissociated when conventional chromatographic methods were used; consequently, the true number of enzyme forms was masked (Dominguez et al., 1992; Sprey and Lambert, 1983). In addition, many endoglucanase isozymes share similar physiochemical properties, such as molecular mass, ionic characteristics, or activitiy toward cellulosic substrates (Enari and Niku-Paavola, 1987). Similarities in physiochemical properties have made the separation of endoglucanases by gel filtration, ion exchange chromatography, or affinity chromatogrphy difficult (Enari and Niku-Paavola, 1987).

The purification of cellulase components has required a combination of successive chromatographic steps that were time consuming and labor intensive (Enari and Niku-Paavola, 1987). Elaborate purification protocols would have benefitted from preliminary information on the cellulase(s) of interest by allowing the design of specific chromatographic and identification methods.

A convenient means for fractionating and identifying

cellulase components would aid studies involving endoglucanase induction and processing, preliminary characterization of new endoglucanase systems, and screening for specific properties (e.g. specificity and molecular mass). A simple and reliable method to identify the spectrum of endoglucanase molecular forms would facilitate their subsequent isolation, purification, and characterization.

Electrophoretic methods used for the fractionation and identification of microbial cellulases

Electrophoresis using nondenaturing conditions The separation of complex protein mixtures is often best achieved by using gel electrophoresis. Kunkel and Tiselius (1952) suggested use of an inert support matrix for electrophoretic separation of peptides to inhibit convection and diffusion disturbances. But available matrices, such as filter paper (Kunkel and Tiselius, 1952) or starch-grain gels (Kunkel and Slater, 1952), did not produce much better resolution than the original free-boundry (reviewed by Tiselius, 1957) electrophoresis method. Resolution was dramatically improved in 1955, however, when Smithies (1955) used starch (partially hydrolysed) as a support gel during electrophoresis. The small pore size of starch gels sufficiently retarded protein molecules so that separation was based on molecular size as well as charge. Starch gels

revolutionized the analyses of protein mixtures; consequently, electrophoresis became standard scientific practice. Polyacrylamide was soon introduced by Ornstein (1964) and Davis (1964) as a suitable gel replacement for starch. Polyacrylamide gels were easy to prepare, thermostable, transparent, and could be used with a variety of pore sizes. Starch gels, however, had poor reproducibility and were difficult to prepare. At present, the support matrix used for electrophoresis is dependent on the application. For example, starch gels have been preferred over polyacrylamide gels when a protocol requires extensive manipulation and slicing of the support matrix after electrophoresis (Selander and Levin, 1980).

In 1958, Hunter and Markert demonstrated that mouse liver esterase activity could be directly detected in starch gel columns after electrophoresis. Starch gel cylinders that contained the separated liver enzyme extracts were soaked in a substrate solution containing α-naphthylbutyrate and a dye coupler. Using this method, Hunter and Markert (1958) were able to detect esterase activity as distinct colored bands in the gel. This "zymogram" method, as it was termed, provided a new means for analyzing the enzymatic composition of biological materials.

Zymogram procedures were soon developed by population geneticists to study enzyme variation in bacteria (Ochman et

al, 1983; Selander and Levin, 1980) and higher organisms (Lewontin, 1974; Lewontin and Hubby, 1966). Several investigators have examined bacterial relationships with a zymogram method referred to as multilocus enzyme electrophoresis (Ochman et al., 1983; Selander and Levin, 1980). Using this method, Ochman et al. (1983) identified allelic enzyme varients (allozymes) in bacteria after simple electrophoresis by "staining" the starch gel support matrix with specific substrates. Because many enzyme samples can be examined at one time using multilocus electrophoresis, large-scale studies estimating genetic diversity of numerous bacterial species have been performed (Ochman et al., 1983; Selander and Levin, 1980). Bacterial enzyme activities that have been detected by using multilocus electrophoresis include hydrolases, isomerases, lyases, oxidoreductases, and transferases (Selander et al, 1986).

Erikkson and Pettersson (1973) were the first to adapt a zymogram method for the identification of microbial cellulase isozymes after separation by polyacrylamide gel electrophoresis. Their enzyme detection method involved spraying a CMC substrate solution onto the polyacrylamide gel and incubating the matrix at 35°C to allow for enzyme action. After incubation, filter paper was placed on the gel to absorb released reducing sugars that resulted from CMC hydrolysis. Using the chromogen p-anisidine and H_2O_2,

reducing sugars were detected on the filter paper as distinct colored bands that indicated the sites of CMCase activity. Although endoglucanase forms were detected using this method, it was time consuming and lacked sensitivity (Beguin, 1983; Schwarz, 1987). Since the Erikkson and Pettersson (1973) technique was developed, many other zymogram methods for the identification cellulase components have been introduced.

Most of the current zymogram methods for detecting cellulase activity have used substrate-laden agar replicas after polyacrylamide gel electrophoresis (Bartley et al, 1984; Biely et al, 1985; McHale and Coughlan, 1981; Nummi et al., 1980). The substrate-agar replicas were overlaid onto polyacrylamide gels and incubated to permit the isolated cellulases to diffuse into the substrate matrix and engage in enzyme action. The replicas were removed from the polyacrylamide gels once hydrolysis zones became evident or after a predetermined time. The cellulose substrates that have been used in agar replicas include amorphous cellulose forms (Nummi et al, 1980), cellulose-azure (McHale and Coughlan, 1981), CMC (Bartley et al., 1984), or HEC-ostazin-brilliant-red (Biely et al., 1988). Hydrolysis of amorphous cellulose in agar replicas reveals clear activity zones against an opaque background (Nummi et al., 1980). Cellulose-Azure is an insoluble, azure-dyed, crystalline

substrate; upon hydrolysis, soluble, azure-labeled-cellooligosaccharides are released (McHale and Coughlan, 1981). In a replica, the soluble products diffuse into the electrophoresis support matrix, resulting in azure-labeled (blue) bands that indicate the sites of cellulase activity (McHale and Coughlan, 1981). Activity zones in CMC-embedded agar replicas can be detected by staining unhydrolyzed substrate in the gel with potassium iodide (Erikkson and Pettersson, 1973), fluorochromes (Cote et al., 1991), or congo red (Beguin, 1983). Teather and Wood (1982) demonstrated that congo red had an affinity for ß-glucans, which provided the basis of a convenient and sensitive assay system for endoglucanase activity. Hydrolyzed CMC zones in a congo red stained gel are pale red against a dark red background. MacKenzie and Williams (1984) were able to detect 1 ng of active cellulase protein in 5 min with the congo red activity stain. HEC-ostazin brilliant red (HEC-OBR) is a soluble cellulose derivative that was labeled with a red dye. Endoglucanase action in replica gels incorporated with HEC-OBR result in the formation of clear zones revealed aganist a red background. Replica staining procedures are not required when HEC-OBR is used as the substrate; however, congo red methods are more sensitive (MacKenzie and Williams, 1984). Endoglucanase activity has recently been detected in electrophoresis gels by soaking

the matrix in a substrate solution consisting of 5-bromoindoxyl-ß-D-cellobioside (Chernoglazov et al., 1989). Upon hydrolysis of the substrate, 5-bromoindoxyl is released and undergoes oxidation by nitroblue monoterazolium to form colored activity bands in the gel. Although this method precludes the nescessity for an agar replica, it is not as sensitive as the congo red stain. van Tilbeurgh and Claeyssens (1985) distinguished between endoglucanase and exoglucanase activities in polyacrylamide gels after electrophoresis by using a synthetic low molecular mass fluorogenic substrate. Endoglucanase I and cellobiohydrolase I from *T. reesei* were able to hydrolyze 4-methylumbelliferyl-ß-D-cellobioside and release the umbelliferone fluorophore. Cellobiohydrolase I, however, was inhibited by cellobiose. By incubating one section of a polyacrylamide gel in the substrate solution without cellobiose and incubating another section with cellobiose, van Tilberugh and Claeyssens (1985) were able to assign the fluorescent activity bands as resulting from endoglucanase I or cellobiohydrolase I. Endoglucanase II from *T. reesei* also was detected in gels, by using the substrate 4-methylumbelliferyl-ß-D-cellotrioside. Endoglucanase II was the only enzyme from *T. reesei* that gave a visable fluorescent reaction upon hydrolysis of the substrate.

Electrophoresis using denaturing conditions

Electrophoretic separations using nondenaturing conditions ensures that enzymes will remain active in the gel for zymogram detection (Gabriel and Gersten, 1992). Nondenaturing electrophoretic conditions, however, may not sufficiently resolve cellulase aggregates into monomeric units (Lamed et al., 1983; Sprey and Lambert, 1983), and molecular mass estimates of protein or activity bands cannot be assigned (Hames and Rickwood, 1981). Sprey and Lambert (1983) isolated a cellulase complex that was purified by isoelectric focusing and other methods. Although the protein complex formed one homogeneous band when it was refocused, it exhibited endoglucanase, ß-glucosidase, and xylanase activities. Further separation of this enzyme complex in the presence of 6 M urea and a mild detergent revealed at least six protein bands. Three of the bands were subsequently identified as an endoglucanase, a ß-glucosidase, and a xylanase by using zymogram analysis. Sprey and Lambert (1983) concluded that the homogeneous complex purified by isoelectric focusing was actually a protein aggregate consisting of many different enzymes and unidentified components that could not be dissociated using nondenaturing conditions. They also suggested that the protein complex was electrofocused at a pI totaling the net charges of the individual components. Large macromolecular

cellulase complexes have been frequently isolated from the culture filtrates of *C. thermocellum* using gel filtration and nondenaturing electrophoresis (Hon-Nami et al., 1986; Lamed et al., 1983; and Mayer et al., 1987). But studies have shown that the cellulase complexes can be electrophoretically dissociated into a plethora of enzymatically active species after treating the aggregates with heat in the presence of SDS (Lamed et al., 1983 and Mayer et al., 1987). Some of the enzymatically active species possessed endoglucanase activity. Saddler and Khan (1980) isolated a 33-kDa endoglucanase from *Acetivibrio cellululyticus* by using nondissociating electrophoretic conditions. The 33-kDa enzyme, however, was futher fractionated by electrophoresis into 10.4-kDa CMC active components after heat treatment in the presence of SDS and 2-mercaptoethanol. Saddler and Khan (1980) hypothesized that the 33-kDa endoglucanase was actually a trimer of the 10.4-kDa species. In addition to insufficient dissociation of cellulase complexes, nondenaturing electrophoresis cannot be used to estimate molecular masses of enzymes, since separation is based on molecular charge as well as size (Hames and Rickwood, 1981). Endoglucanases, however, are often characterized by their molecular mass (Enari and Niku-Paavola, 1987). Separation protocols using denaturing conditions may eliminate problems sometimes associated with

nondenaturing electrophoresis systems. Heat treatment in the presence of SDS effectively dissociated cellulase aggregates and multimers into individual components which were separated by SDS-PAGE (Lamed et al., 1983; Saddler and Khan, 1980; Sprey and Lambert, 1987).

Antibodies have recently been used to detect cellulase forms after separation by denaturing SDS-PAGE and western blotting (Luderer et al., 1991; Messner et al., 1988). Monoclonal antibodies prepared against endoglucanase I, cellobiohydrolase I, and ß-glucosidase identified cellulase molecular forms in commercial and laboratory enzyme preparations (Luderer et al., 1991; Messner et al., 1988). But molecular mass values could only be assigned to protein bands that specifically reacted with the antibodies, and enzyme activity could not be directly assayed in the system used.

In 1977, Rosenthal and Lacks described an enzyme detection method that combined denaturing SDS-PAGE with zymogram activity analysis. SDS-PAGE has been used to separate proteins according to their molecular mass (Hames and Rickwood, 1981). The method is based on the ability of the anionic detergent SDS to bind to protein molecules in a constant ratio of 1.4 to 1.0. SDS saturates proteins with negative charges, thus minimizing any affects of the net charges that exist on the polypeptides. Consequently,

separation occurs only by sieving through the polyacrylamide matrix. In the Rosenthal and Lacks (1977) study, purified and crude extracts of bacterial nucleases were denatured with heat in the presence of SDS and separated by SDS-PAGE. The nucleases were subsequently renatured in the gel after incubation in buffer to remove the SDS. DNA or RNA substrates were embedded in the separating gel before electrophoresis. Nuclease activities in the gels were detected as dark hydrolysis zones or bands appearing against fluorescent, ethidium-bromide-stained backgrounds. Rosenthal and Lacks (1977) demonstrated that SDS was sufficiently removed from the system to allow enzyme renaturation, and that a multiplicity of nuclease activities were present in extracts from *B. subtilis*, *Escherichia coli*, and *Haemophilus influenzae*.

Not all microbial enzymes were amenable, however, to denaturing SDS-PAGE zymogram analysis. In a study by Lacks and Springhorn (1980), oligomeric enzymes composed of subunits with different molecular masses were not renatured in polyacrylamide gels after heat treatment in the presence of SDS prior to SDS-PAGE and zymogram analyses. Oligomeric enzymes consisting of identical subunits were only poorly renaturable. Lacks and Springhorn (1983) suggested that the loss of cofactors required for proper subunit orientation or catalysis may have contributed to failure of the oligomeric

enzymes with identical subunits to renature. Most of the monomeric enzymes tested, except trypsin, were able to regain activity in gels, even when 2-mercaptoethanol was added to the denaturing treatment before SDS-PAGE. By using the denaturing zymogram method, molecular mass estimates of the monomeric enzymes tested corresponded to the molecular mass values of the native proteins.

Beguin (1983), Lamed et al. (1983), and Schwarz (1987), developed denaturing zymogram methods to study the endoglucanases produced by *C. thermocellum*. The enzymes were good candidates for denaturing zymogram analyses because they are monomeric (Hazelwood et al., 1988). In the Beguin (1983) and Lamed et al. (1983) studies, cellulase *C. thermocellum* culture filtrates were denatured with heat (60°C for 60 min) in the presence of SDS (3% final concentration) and then were fractionated by SDS-PAGE. After electrophoresis, endoglucanases were detected in the separating gels by using CMC-laden agar replicas as described in the preceeding section. By using this method, Beguin (1983) and Lamed et al. (1983) demonstrated the existence of multiple endoglucanase forms that were all active on the substrate CMC. Agar replica methods for endoglucanase detection, however, have suffered from low sensitivity because of slow and inefficient diffusion of renatured enzymes from small-porosity polyacrylamide gels

(Beguin et al., 1983; Schwarz et al., 1987). Schwarz et al. (1987) improved the detection sensitivity by incorporating the CMC substrate into the separating gel. Since CMC was embedded in the polyacrylamide gel, enzyme diffusion was not required for activity detection. Loss of enzyme activity due to insufficient diffusion was avoided and sensitivity was increased. This *in situ* denaturing zymogram method improved endoglucanase detection sensitivity by two orders of magnitude over the replica method used by Beguin (1983) and Lamed et al. (1983). In addition to the increased sensitivity, Schwarz (1987) was able to simultaneously detect CMCase and 4-methylumbelliferyl-ß-D-cellobiosidase activities in the same gel by using protocols previously described for the substrates in the paragraph headed "Electrophoresis using nondenaturing conditions".

Denaturing zymogram analysis combined the advantages of enzyme identification with the resolution and molecular mass dependence of SDS-PAGE (Lacks and Springhorn, 1980). Many endoglucanase forms were detected from crude and purified enzyme preparations, providing information on molecular mass, endoglucanase complement, substrate specificity, and sample purity (Beguin, 1983; Lamed et al., 1983; and Schwarz et al., 1987). Preliminary knowledge of endoglucanase systems would provide direction for more elaborate and efficient isolation, purification, and characterization

protocols.

The Schwarz (1987) *in situ* zymogram technique is a simple and sensitive method to detect endoglucanase forms from the anaerobic thermophile, *C. thermocellum*. But further evaluation of its reliability and compatibility with cellulase systems from other microorganisms is needed.

Cote et al. (1991) recently demonstrated that enzyme extracts from a *Trichoderma* sp. and other fungi regained activity after denaturing SDS-PAGE analysis. 1,3-Glucanase (lichenenase) activities were detected in separating gels embedded with lichenen prior to electrophoresis.

MATERIALS AND METHODS

Protein and Enzyme Activity Measurements.

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. Protein was measured by a Coomassie Brilliant Blue G-250 dye-binding assay (Bio-Rad Protein Assay, Bio-Rad, Richmond, CA). Bovine serum albumin was used as the protein standard.

Carboxymethylcellulase (CMCase or endo-1,4-ß-glucanase) activity was measured by using a copper-bicinchoninate reducing sugar test (Waffenschmidt and Jaenicke, 1987) as modified by Fox and Robyt (1991). A copper-bicinchoninate working reagent was prepared daily by mixing two stock solutions in equal volumes. Stock solution A consisted of 2,2'-bicinchoninate dissolved in a solution of 3.2 g $Na_2CO_3H_2O$ and 1.2 g $NaHCO_3 \cdot H_2O$ in 45 ml dH_2O and adjusted to 50 ml. Stock solution B consisted of 62 mg of $CuSO_4 \cdot 5H_2O$ and 63 mg of L-serine dissolved in 45 ml of dH_2O and adjusted to 50 ml. The substrate solution consisted of 0.5% low-viscosity carboxymethylcellulose (CMC, Fluka Chemical Corp. Ronkonkoma, NY) in sodium acetate buffer (0.05 M, pH 5.0) containing 0.02% sodium azide. The reaction mixture consisted of 0.2 ml of enzyme preparation and 4.8 ml of substrate solution. The reaction mixture was incubated in

25 ml Erlenmyer flasks for 20 minutes at 50°C while being mixed at 150 rpm in a reciprocating water bath (Orbit Microprocessor Shaker Bath, Lab-Line Insruments Inc. Melrose Park, IL). Samples (0.3 ml) were removed at 2-minute intervals. Each sample was mixed with an equal volume of copper-bicinchoninate working reagent and placed into a well of a microtitration plate (Immulon 1, flat bottom plates, Dynatech Laboratories Inc, Chantilly, VA). Triplicate analyses were performed at each sample time. The plate was covered with a 76 mm x 128 mm strip of transparent tape, and incubated for 35 minutes at 80°C. Absorbance was measured at 560 nm after the plate had cooled at room temperature for 15 minutes. Absorbance values were subtracted from enzyme and substrate blanks to correct for reducing groups attributed to the digest reagents. A glucose standard (0.1 to 1.0 μg) was prepared by plotting glucose concentration versus absorbance at 560 nm. The amounts of reducing sugars released from the digests were determined from the standard curve. One unit of enzyme activity was the amount of enzyme liberating 1 uM of reducing sugars in 1 minutes. Xylanase activity was measured as described for CMCase, except that the substrate solution consisted of 0.5% oat spelt xylan and D-xylose (0.1 to 1.0 μg) was used as the standard.

CMCase and 4-methlyumbelliferyl-ß-D-cellobiosidase (MUCELase) activities were detected in polyacrylamide gels

(PAGs), as described by Schwarz et al. (1987) and by Van Tilbeurgh and Claeyssens (1985). Details regarding enzyme-activity detection in polyacrylamide gels will be presented under the heading "Zymogram Analysis."

Enzyme Production

Trichoderma reesei ATCC 26921 (QM9414) and *Myrothecium verrucaria* (ATCC 9095) were cultured on media described by Mandels and Andreotti (1978) and Whittaker (1952), except that cotton linters (30 g) and Solka Floc (10 g) were replaced with equal weights of purified cellulose from Sigma. The *M. verrucaria* culture medium consisted of 10 g cellulose, 3.8 g $NaNO_3$, 2.0 g $NaH_2PO_4 \cdot H_2O$, 1.5 g Na_2HPO_4, 1.0 g glucose, 0.6 g NH_4NO_3, 0.2 g KH_2PO_4, 0.1 g K_2HPO_4, 2.0 mg $ZnSO_4 \cdot 7H_2O$, 1.2 mg $Ca(NO_3)_2 \cdot 4H_2O$, 0.4 mg $CoCl_2 \cdot 6H_2O$, 0.08 mg $MnCl_2 \cdot 4H_2O$. 0.06 mg; H_3BO_3, 0.05 mg; $FeSO_4 \cdot H_2O$, and 0.04 mg $(NH_4)_6Mo_7O_{24}$ per liter of dH_2O. The pH of the medium was 6.6 after sterilization in an autoclave. The *T. reesei* culture medium consisted of 10 g cellulose, 2.0 g KH_2PO_4, 1.4 g $(NH_4)_2SO_4$, 0.5 g proteose peptone, 0.3 g $CaCl_2$, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.3 g urea, 5.0 mg $FeSO_4 \cdot 7H_2O$, 2.0 mg $CoCl_2$, and 1.4 mg $MnSO_4 \cdot H_2O$ per liter of dH_2O. The pH of the medium after sterilization was 6.0.

Inocula were prepared by adding 10^7 spores per ml of

each fungus to 250-ml Erlenmeyer flasks containing 50 ml of the appropriate culture medium. The flasks were incubated on a reciprocating shaker for 3 days at 30°C and 200 rpm (New Brunswick Scientific Co. New Brunswick, NJ). Fernbach flasks (2.8-L) containing 600 ml of appropriate culture medium were inoculated with the contents of the 250-ml flasks (5% mycelial inoculum) and incubated on a reciprocating shaker for 7-9 days at 30°C. Samples from the Fernbach culture flasks were removed each day to measure protein, enzyme activity, and pH.

Enzyme Preparation

Spent culture fluid from each fungus was clarified by filtration through glass wool. The remaining insoluble material was removed by centrifugation at 10,000 x *g* for 30 minutes at 4°C. Supernatents (500 ml) from each fungus were lyophilized and stored at 4°C until needed. For analysis, a portion (200 mg) of freeze-dried culture filtrate was dissolved in 1 ml of Tris-HCl buffer (0.01 M; pH 6.8), and insoluble material was removed by centrifugation at 16,000 x g for 5 minutes at room temperature (Micro-Centaur Centrifuge, MSE Scientific Instruments, Sussex, England). The enzyme solutions were desalted on a Bio-Gel P-2 column (1 cm x 6 cm), using Tris-HCl buffer, and stored at -100°C

with 5% glycerol until needed. Two commercial cellulase preparations, Rohament CT (derived from *T. reesei;* Rohm Tech Inc., Malden, MA) and *T. viride* type 5 (Sigma) were desalted and stored in the same manner.

Samples of *Bacillus subtilis* endo-1,4-ß-glucanase were obtained from Dr. Gordon Willick [Institute for Biological Sciences, Ottawa, CAN (1991)] as ammonium sulfate precipitates. The protein precipitates were pelleted by centrifugation (10,000 x g) for 5 minutes, the supernatant was discarded, and the pellet was resuspended in Tris-HCl buffer to its original volume. The 52.2 kDa protein was desalted by ultrafiltration (Ultrafree MC polysulphone membrane, 0.4-ml unit, 10 kDa exclusion, Millipore Corp., Bedford, MA) and resuspended to its original volume. The 30-33 kDa protein was precipitated with cold acetone and stored overnight at -20°C. The precipitate was pelleted by centrifugation and resuspended in Tris-HCl to its original volume.

Electrophoresis

SDS-PAGE was performed in polyacrylamide slab gels (16 cm x 16 cm x 1 mm) in the presence of SDS essentially as described by Laemmli (1970). Separating (7%-12% T, 3% C) gels were prepared from the stock solutions listed in Table

1. T denotes the total monomer concentration (acrylamide and bisacrylamide) and C denotes the bisacrylamide concentration relative to the total monomer concentration. The acrylamide stock solution consisted of 29.2% (w/v) acrylamide and 0.8% of N,N'-*bis*-methylene-acrylamide (30% T, 2.67% C). The 10% ammonium persulfate solution was prepared daily. All SDS-polyacrylamide slabs contained 0.1% CMC or xylan incorporated into the separating gels prior to polymerization. The stacking gel (4% T, 3% C) consisted of 6.1 ml dH_2O, 2.5 ml 0.5 M Tris-HCl, pH 6.8, 1.3 ml acrylamide stock, 100 μl 10% SDS, 50 μl 10% ammonium persulfate, and 10 μl TEMED. Separating and stacking solutions were prepared by combining all reagents except the 10% ammonium persulfate solution and TEMED. The mixture was degassed under vacuum for 15 minutes. Polymerization of the separating gel was initiated by adding 500 μl of 10% ammonium persulfate and 50μl of TEMED. The polyacrylamide solution was cast into a vertical glass plate sandwich assembly (Protean II xi electrophoresis unit, Bio-Rad Laboratories, Richmond, CA.) with 1 mm spacer bars and was immediately overlaid with a 0.5-cm layer of water to flatten the gel surface. The acrylamide slab solidified after 1 hour and the final gel dimensions were 14 cm x 16 cm x 1 mm. The water layer was removed after gel solidification and a 15-well comb assembly was attached to the top of the glass

Table 1. Composition of 7.0%-12.0% T, 3% C, separating gels used during SDS-polyacrylamide gel electrophoresis.

	Separating gel concentration % T[a]			
Stock solution (ml)	7.0%	7.5%	8.0%	12.0%
dH_2O	47.1	45.5	43.8	30.4
1.5 M Tris-HCl, pH 8.8	25	25	25	25
Acrylamide (30% T, 3% C)	23.3	25	26.7	40
Glycerol	3	3	3	3
10% SDS (w/v)	1	1	1	1
10% Ammonium persulfate	0.5	0.5	0.5	0.5
TEMED	0.05	0.05	0.05	0.05
CMC or xylan (mg)	100	100	100	100

[a]T denotes the total monomer concentration (acrylamide and bisacrylamide) and C denotes the bisacylamide concentration relative to the total monomer concentration.

plate sandwich. Polymerization of the stacking acrylamide solution was initiated by adding 50 μl of 10% ammonium persulfate and 10 μl of TEMED, then the mixture was poured over the separating gel until the comb wells were totally immersed. The dimensions of the stacking gel were 2 cm x 16 cm x 1 mm. The comb assembly was removed after gel solidification (1 hour) and the entire sandwich assembly, which harbored the complete polyacrylamide slab, was clamped onto the electrophoresis unit. The electrode buffer (pH 8.3) was prepared as a 5X solution and contained 72 g glycine, 15 g Tris base, 5 g SDS and dH_2O to 1 liter. Electrode buffer was added to the upper (cathode; 400 ml) and lower (anode; 1800 ml) buffer chambers. Enzyme samples containing 1-30 μg in 20-μl volumes, were mixed with an equal volume of 2X sample buffer and the mixture was heated at 95°C for 5 minutes. The sample buffer (0.12 M Tris-HCl; pH 6.8) was prepared by dissolving 3 g of SDS and 5 ml of glycerol in 45 ml of 0.12 M Tris. The pH was adjusted to 6.8 with concentrated HCl and the final sample buffer volume was adjusted to 50 ml. A reducing agent was not incorporated into the sample buffer. Molecular mass protein standards were prepared as 1 mg/ml stock solutions in 0.06 M Tris-HCl buffer, pH 6.8. The protein markers were mixed (4 μl each) with 20 μl of 2X sample buffer that contained 2% 2-mercaptoethanol, and were heated at 95°C for 5 minutes.

Molecular mass markers (kDa) used were phosphorylase B (97.4), bovine serum albumin (66), egg albumin (45), carbonic anhydrase (29), and trypsin inhibitor (20). Four microliters of 0.8% m-cresol purple were added to each 40-μl sample as the tracking dye. Enzyme samples and protein markers were loaded into the stacking gel wells. Sample buffer diluted to 1X with dH_2O was added to each blank well. The cooling core of the electrophoresis unit was filled with an ethylene glycol:dH_2O mixture in the ratio of 20:80 as a coolant. Electrophoresis was performed at room temperature using constant current conditions (Model 494 electrophoresis power supply, Isco, Inc, Lincoln, NE). A current of 16 mA was used during sample migration through the stacking gel and 20 mA during sample migration through the separating gel. Electorphoresis was halted when the tracking dye reached 1 cm from the gel bottom (about 4 hour). Protein bands were detected with a silver-stain kit (Sigma). Molecular mass standard curves were prepared for each electrophoresis run by plotting the log of the molecular mass versus the migration (cm) of the protein markers from the top of the separating gel (Hames and Rickwood, 1981). Phosphorylase b (97.4 kDa) was not used to prepare the molecular-mass standard-curves because the protein migrated outside the linear molecular mass range of the gel concentrations used (Hames and Rickwood, 1981). Molecular

mass estimates were confined between 20-66 kDa.

Zymogram Analyses

After separation of the enzyme samples by means of SDS-PAGE, CMCase, MUCELase, and xylanase activities were detected directly in the polyacrylamide gels. CMCase activity was detected essentially as described by Schwarz et al. (1987) except that 10 mM dithiothreitol in 0.1 M succinate buffer was not used to wash the gels. After electrophoresis, the gels were washed for 1 hour with 3 changes of distilled water, soaked for 10 minutes in sodium acetate buffer at room temperature and incubated for 45 minutes at 45°C. The acidic gels were neutralized with Tris-HCl (0.1 M; pH 9.0) for 20 minutes and stained with 0.1% congo red solution in water for 30 minutes. Pale red hydolysis zones (CMCase zones) emerged against a red background after destaining with 1 M NaCl. Contrast enhancement of the hydrolysis zones was achieved by soaking the gel in a protein-fixative solution (50% methanol:10% acetic acid:dH_2O) for 10 minutes. MUCELase activity was detected as described by van Tilbuergh and Claeyssens (1985). After electrophoresis, the gels were washed as described brfore, except that 0.5 mM MUCEL was added to the sodium acetate buffer and the preparation was incubated

immediately. In some instances, cellobiose (5 mM) and gluconolactone (1 mM) were added to the sodium acetate buffer to inhibit ß-glucosidase or cellobiohydrolase I activities, which may act on MUCEL (Van Tilbeurgh and Claeyssens, 1985). Fluorescent zones of MUCELase activity were visualized with a shortwave UV transilluminator. Wells were removed at the precise locations of the fluorescent bands to mark the bands so that molecular mass estimates could be assigned to the active zones. Xylanase activity was detected in gels as described for CMCase activity except that the CMC was replaced with 0.1% xylan in the separating gel.

Molecular mass markers, protein content, and activity patterns could all be detected in the same gel by dividing it into sections after electrophoresis. After protein and activity pattern development, the gel sections were brought back together so that direct comparisons could be made and molecular mass estimates could be assigned to each zone or band. The gels were photographed under white light by using Polaroid Type 55 film.

RESULTS

Enzyme Production

Parameters measured during extracellular CMCase production by *T. reesei* are summarized in Table 2. An extracellular protein concentration of 8 ug/ml and CMCase activity of 0.2 U/ml were detected after incubation for only 1 day at 30°C. The largest daily increases in protein concentration (16 ug/ml/day) occurred between days 4 and 5 and days 5 and 6. The smallest increase in protein concentration (2 ug/ml) occurred between days 6 and 7. The largest increase in CMCase activity (2.4 U/ml) occurred between days 1 and 2 and the lowest increase in activity (0 U/ml) occurred between days 5 and 6. The final protein concentration and CMCase activity measurements were 71 ug/ml and 4.8 U/ml, respectively, when the culture fluid was harvested on day 7. The specific activity peaked at 153 U/mg on day 2 and steadily decreased to 68 U/mg on day 7.

Parameters measured during *M. verrucaria* enzyme production is summarized in Table 3. An extracellular protein concentration of 5 μg/ml was detected on day 1 ;however, CMCase activity was not detected until day 3 (0.5 U/ml). The largest increase in protein concentration (22 ug/ml) occurred between days 8 and 9, and the lowest

Table 2. *T. reesei* enzyme (CMCase) production summary.

Incubation (days)	Protein[a] (mg x 10^{-3}/ml)	Activity[b] (U/ml)	Specific activity (U/mg)
0	0	0	0
1	8	0.2	25
2	17	2.6	153
3	24	3.3	137
4	37	4.2	113
5	53	4.5	85
6	69	4.5	65
7	71	4.8	68

[a]Protein concentration was determined by using a Coommassie Brilliant Blue G-250 dye-binding assay.

[b]CMCase activity was determined by measuring the release of reducing sugars from a CMC substrate solution.

Table 3. *M. verrucaria* enzyme (CMCase) production summary.

Incubation (days)	Protein[a] (mg x 10^{-3}/ml)	Activity[b] (U/ml)	Specific activity (U/mg)
0	0	0	0
1	5	0	0
2	10	0	0
3	28	0.5	18
4	39	0.9	23
5	42	0.8	19
6	55	1.1	20
7	69	1.4	20
8	73	1.7	23
9	95	1.6	17

[a]Protein concentration was determined by using a Coommassie Brilliant Blue G-250 dye-binding assay.

[b]CMCase activity was determined by measuring the release of reducing sugars from a CMC substrate solution.

increases were detected on days 1 and 2 (5 ug/ml). The largest increase in CMCase activity (0.5 U/ml) occurred between days 2 and 3. Slight decreases in activity were recorded on days 5 and 9. The final protein and activity measurements were 95 ug/ml and 1.6 U/ml when the culture filtrate was harvested on day 9, respectively. The specific activity remained relatively constant from day 3 (18 U/mg protein) to day 9 (17 U/mg protein).

Enzyme Preparation

Protein, activity, and specific activity measurements of enzyme preparations used in this study are summarized in Table 4. The *T. reesei* QM9414 culture filtrate protein was concentrated 8-fold, after freeze-drying, from 71 ug/ml (Table 2) to 0.6 mg/ml (Table 4). The CMCase activity was concentrated 12-fold, from 4.8 U/ml to 60 U/ml. The *M. verrucaria* culture filtrate protein and CMCase activities were concentrated 7-fold, from 95 ug/ml (Table 3) to 0.7 mg/ml (Table 4) and 12-fold, from 1.6 U/ml to 19 U/ml. The commercial cellulase preparations (*T. reesei*, Rohm Tech and *T. viride*, Sigma) had the highest protein concentrations (3.8 and 4.0 mg/ml) and CMCase activities (619 U/ml and 1000 U/ml) were recorded; however, their specific activities (163 U/mg and 250 U/mg) were less than that of the *B. subtilis*

Table 4. Cellulase (CMCase) preparations used in this study.

Source	Protein[a] (mg/ml)	Activity[b] (U/ml)	Specific activity (U/mg)
B. subtilis			
52.2 kDa	0.6	374	623
33 kDa	0.6	416	693
M. verrucaria ATCC 9095	0.7	19	27
T. reesei QM9414	0.6	60	100
T. reesei Rohm Tech	3.8	619	163
T. viride Sigma	4.0	1000	250

[a]Protein concentration was determined by using a Coommassie Brilliant Blue G-250 dye-binding assay.

[b]CMCase activity was determined by measuring the release of reducing sugars from a CMC substrate solution.

52.2 kDa (623U/mg) and 30-33 kDa (693 U/mg) endoglucanase preparations. The 52.2 kDa and 30-33 kDa endoglucanase samples from *B. subtilis* had protein concentrations of 0.6 mg/ml and the CMCase activities were 374 U/ml and 416 U/ml, respectively.

The xylanase activity from the *T. reesei* QM9414 enzyme preparation was 100 U/ml and the specific activity was 167 U/mg (data not shown).

Zymogram Analyses

General conditions for zymogram analyses

No discernable differences in protein banding or CMCase zymogram pattern were detected when enzyme samples were denatured with the following temperature-time combinations: 60°C for 30 minutes, 70°C for 10 minutes, 80°C for 10 minutes, or 95°C for 5 minutes (data not shown). All denaturation treatments were performed in the presence of 3% SDS before electrophoresis. Because it was most convenient, the 95°C, 5 minutes denaturation treatment was used for all subsequent zymogram analyses. No difference in the CMCase zymogram pattern was observed when separating gels were washed with dH_2O for 1 hour at room temperature after electrophoresis instead of with succinate buffer (0.1 M; pH 5.8) containing 10 mM dithiothreitol for 30 minutes as

described by Schwarz et al. (1987, data not shown).

Zymogram analyses of the *T. reesei* cellulase preparation

CMCase zymogram analysis of a serially diluted enzyme sample from *T. reesei* QM9414 is shown in Figure 1. Enzyme samples were denatured in 3% SDS (final concentration) at 95°C for 5 minutes and were subjected to 7.5% SDS-PAGE. CMCase activity was detected in the gel as described in "Materials and Methods" except that the gel was incubated at 45°C for 30 minutes instead of 45 minutes. The protein pattern from the *T. reesei* enzyme sample is displayed in lane 2. A large CMCase activity band was observed in the 45-58 kDa molecular mass region in lane 3, Figure 1. However, a distinct 56 kDa CMCase band was evident in lanes 5-7 as the enzyme sample was diluted. Other distinct CMCase bands evident in lane 3 were assigned molecular mass values of 44, 36, 34, 27, and 25 kDa, respectively. The 44, 36, and 34, and 25 kDa CMCase bands were not observed in lanes 4-8. A 27 kDa CMCase band was evident in lanes 3-5. In all, 6 bands of activity were observed.

CMCase and MUCELase zymogram analysis of the extracellular cellulase preparation from *T. reesei* QM9414 is shown in Figure 2. All enzyme samples were denatured in 3% SDS (final concentration) at 95°C for 5 minutes and were subjected to 8.0% SDS-PAGE. CMCase activity is represented by clearing zones. Bands of MUCELase activity were marked

Figure 1. CMCase zymogram analysis of a serially diluted enzyme sample from *T. reesei* QM9414. Samples were denatured in 3% SDS (final concentration) at 95°C for 5 minutes and were subjected to 7.5% SDS-PAGE. The separating gel contained 0.1% CMC. After electrophoresis, the gel was divided into 2 sections that consisted of lanes 1-2 and lanes 3-8. Lanes 1 (molecular mass markers) and 2 (enzyme), were silver-stained for protein. Values (kDa) of protein molecular mass markers in lane 1 are on the left. Lanes 3-8 (serially diluted enzyme) were stained for CMCase activity with 0.1% congo red as described in "Materials and Methods". The following amounts of enzyme sample were used; lane 2, 15 μg (1.5 U); lane 3, 4 μg (0.4 U); lane 4, 2 μg (0.2 U); lane 5, 1 μg (0.1 U); lane 6, 0.5 μg (0.05 U); lane 7, 0.2 μg (0.02 U); lane 8, 0.1 μg (0.01 U). Molecular mass estimates (kDa) of enzyme activity bands are on the right.

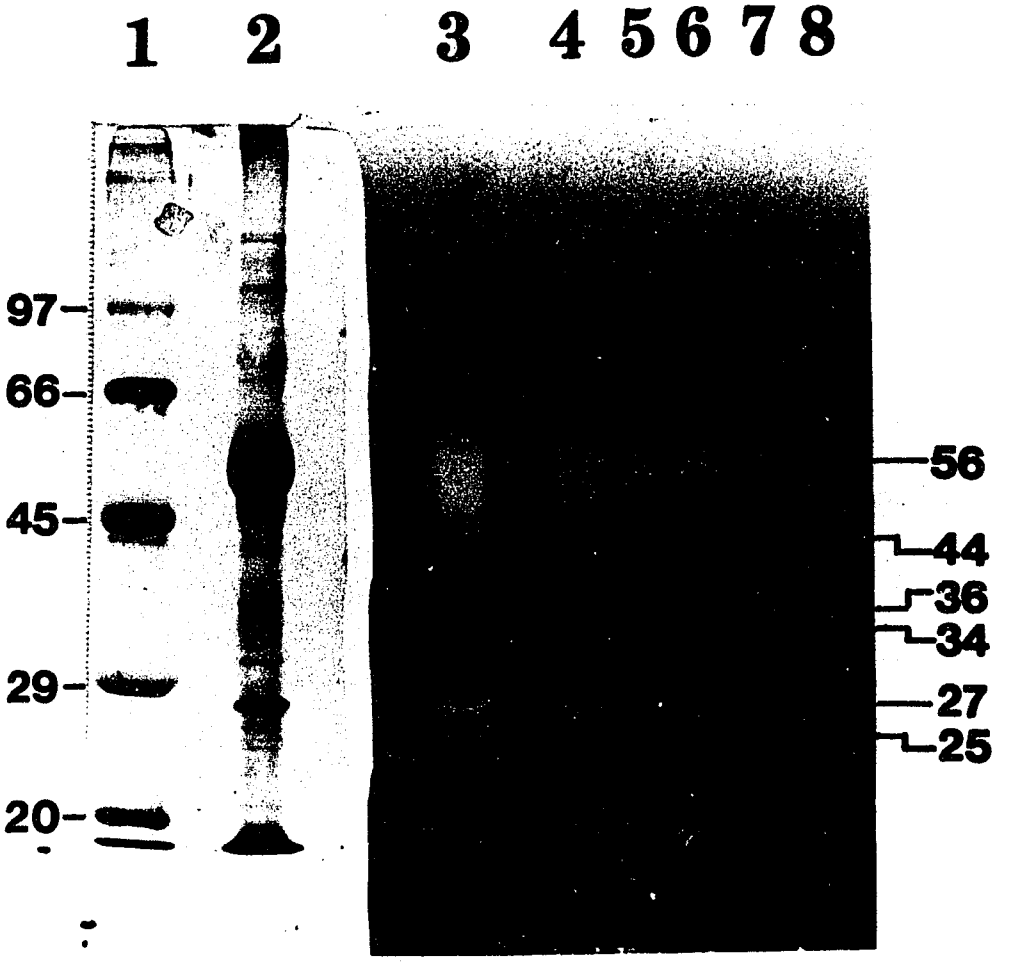

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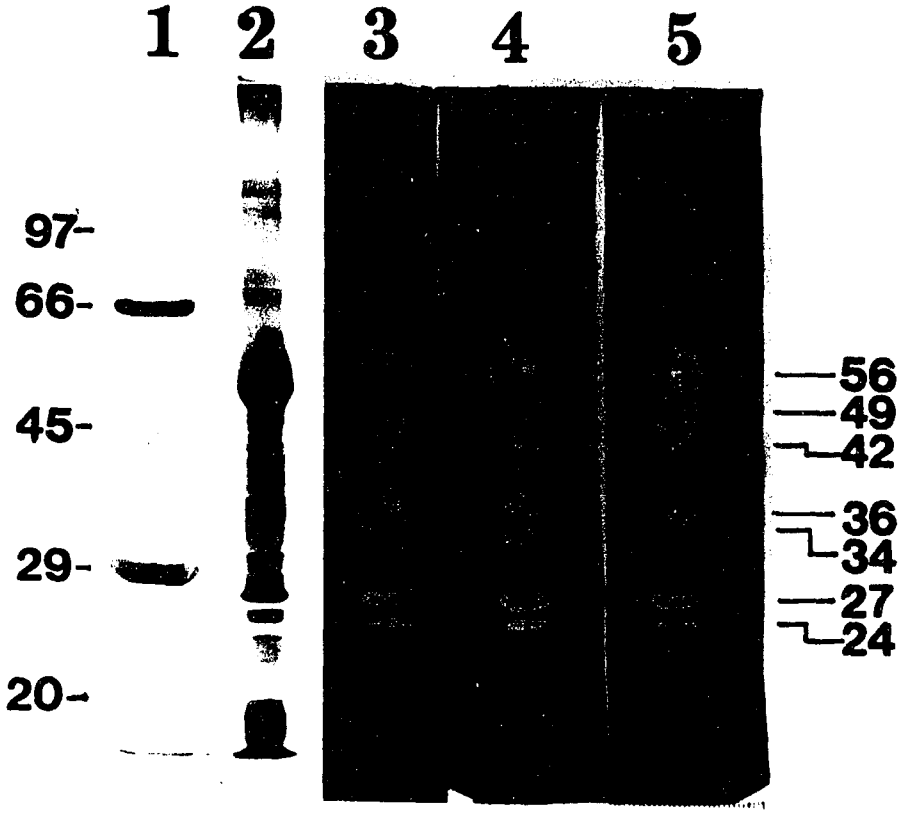
Figure 2. Zymogram analysis of a cellulase preparation from *T. reesei* QM9414. Samples were denatured in 3% SDS at 95°C for 5 minutes and were subjected to 8.0% SDS-PAGE. After electrophoresis, the gel was divided into 4 sections that consisted of lanes 1 and 2, lane 3, lane 4, and lane 5. Lanes 1 and 2 were silver-stained for protein. Values (kDa) of protein molecular mass markers in lane 1 are on the left. Lane 3 (containing enzyme) was stained for CMCase activity. Lanes 4 and 5 (containing enzyme) were both stained for CMCase and MUCELase activity. Lane 4 did not contain enzyme inhibitors in the MUCEL substrate solution. Lane 5 contained cellobiose (5mM) and gluconolactone (1mM) in the MUCEL substrate solution. CMCase activity bands are represented as clearing zones in the gel. Fluorescent MUCELase activity bands were visualized under short-wave UV-light and their positions marked with a holepunch. The following amounts of enzyme sample were used: lane 2, 15 μg (1.5 U); lanes 3, 4, and 5, 4 μg (0.4 U). Molecular mass (kDa) estimates of enzyme activity bands are on the right.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by using a holepunch; these appear as light spots in the figure. The protein pattern from the *T. reesei* enzyme sample is shown in lane 2. The CMCase activity pattern in lanes 3-5 are similar except that the clearing zones in lane 4 are more pronounced due to an extended incubation time of 10 minutes at 45°C. A large amount of CMCase activity was observed in the 44-58 kDa molecular mass region (lanes 3-5), and a 56-kDa MUCELase activity band was observed in lanes 4 and 5. MUCELase activity was not observed at the 49-kDa molecular mass region. Other prominent CMCase activity bands observed in lane 5 were assigned molecular mass values of 42, 36, 34, 27, and 24 kDa, respectively. Less prominent CMCase activity bands at 28 kDa, 37-41 kDa, and a very faint band near the 97 kDa marker were most evident in lane 4. The prominent CMCase and MUCELase components identified in Figure 2, lane 5, are summarized in Table 5.

Zymogram analysis of different quantities of a *T. reesei* QM9414 enzyme preparation using xylan as the substrate is shown in Figure 3. The separating gel contained 0.1% xylan. Enzyme samples were denatured in 3% SDS (final concentration) at 95°C for 5 minutes and were subjected to 7.0% SDS-PAGE. Prominent xylanase activity bands were observed in lane 2 at 55, 47, 44, 40, 22, and 20 kDa. A less prominent xylanase activity band was observed near the 97 kDa molecular mass marker. Five very faint

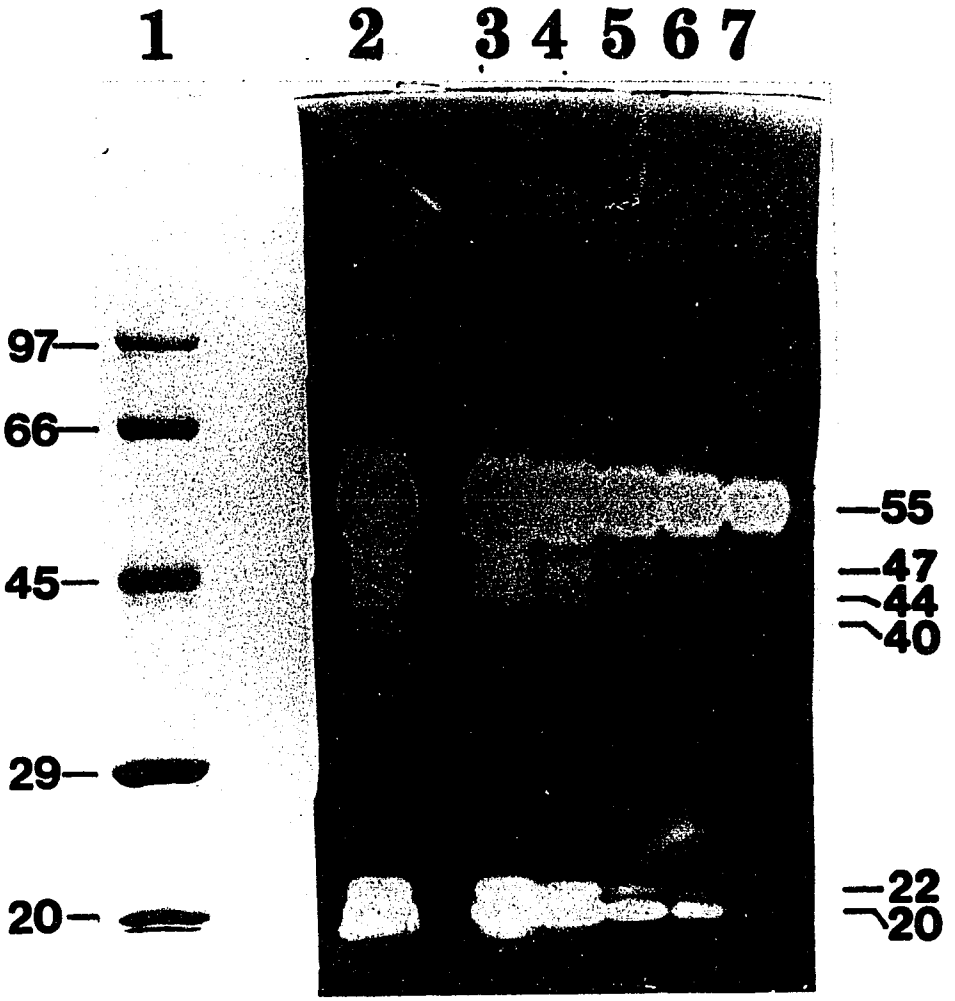
Table 5. Major cellulase components from *T. reesei* QM9414 identified by zymogram (CMCase and MUCELase) analysis.

Molecular mass estimate (kDa)	CMCase[a]	MUCELase[b]
56	+	+
49	+	-
42	+	-
36	+	-
34	+	-
27	+	-
24	+	-

[a]CMCase activity was detected by staining the separating gel that contained CMC with congo red after electrophoresis of an enzyme sample.

[b]MUCELase activity was detected by flooding the separating gel with MUCEL substrate solution after electrophoresis of an enzyme sample.

Figure 3. Zymogram (xylanase) analysis of a *T. reesei* QM9414 enzyme preparation with 0.1% xylan incorporated into the separating gel. Samples were denatured in 3% SDS at 95°C for 5 minutes and were subjected to 7.0% SDS-PAGE. After electrophoresis, the gel was divided into 2 sections that consisted of lane 1 (molecular mass proteins) and lanes 2-7 (enzyme). Lane 1 was silver-stained for protein. Values (kDa) of protein molecular mass markers in lane 1 are on the left. Lanes 2-7 were stained for xylanase activity using 0.1% congo red as described in "Materials and Methods". The following amounts of enzyme sample were used; lane 2, 8 μg (1.3 U); lane 3, 4 μg (0.6 U); lane 4, 2 μg (0.3 U); lane 5, 1 μg (0.15 U); lane 6, 0.5 μg (0.07 U); lane 7, 0.2 μg (0.03 U). Molecular mass estimates (kDa) of xylanase activity bands are on the right.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20

activity bands also were displayed between the 23-40 molecular mass region. In all, 12 bands of activity were observed.

Zymogram analyses of the *M. verrucaria* cellulase preparation

CMCase and MUCELase zymogram analysis of a serially diluted enzyme sample from *M. verrucaria* ATCC 9095 is shown in Figure 4. Samples were denatured in 3% SDS (final concentration) at 95°C for 5 minutes and were subjected to 8.0% SDS-PAGE. The protein pattern of the *M. verrucaria* enzyme sample is shown in lane 2. Lane 3 shows CMCase activity bands (clearing zones) and MUCELase activity bands (light circular areas; marked with a holepunch). Lanes 4-8 show CMCase activity only. The prominent CMCase bands were assigned values of 64, 49, 43, and 26 kDa. A 26 kDa protein band in lane 2 was not detected. Two faint CMCase bands were observed near the 100 kDa molecular mass region in lane 4 that were less evident in lane 3. Lane 3 contained 7 μg of protein and lane 4 contained 8 μg of protein. MUCELase activity bands were observed at 64 kDa and 49 kDa, respectively (lane 3). The 64-kDa MUCELase activity band was marked with a holepunch in the center of the fluorescent band (lane 3). The 49-kDa MUCELase band was marked with a holepunch at three positions to determine the length of the fluorescent band (lane 3). The prominent activity bands displayed in Figure 4 are summarized in Table 6.

Figure 4. Zymogram analysis of an enzyme sample from *M. verrucaria* ATCC 9095. Samples were denatured in 3% SDS at 95°C for 5 minutes and were subjected to 8.0% SDS-PAGE. The separating gel contained 0.1% CMC. After electrophoresis, the gel was divided into 2 sections that consisted of lanes 1 and 2 and lanes 3-8. Lanes 1 and 2 were silver-stained for protein. Values (kDa) of protein molecular mass markers in lane 1 are on the left. Lanes 3-8 were stained for CMCase and MUCELase activity. CMCase activity (represented as clearing zones) was detected with 0.1% congo red. MUCELase activity was detected as described in "Materials and Methods. No enzyme inhibitors were added to the MUCEL substrate solution. Fluorescent MUCELase activity bands were visualized under short-wave UV-light and their positions marked with a holepunch. Only lane 3 was marked for MUCELase activity. The following amounts of enzyme sample were used: lanes 2 and 3, 7 μg (0.19 U); lane 4, 8 μg (0.2 U); lane 5, 4 μg (0.1 U); lane 6, 2 μg (0.05 U); lane 7, 1 μg (0.02 U); lane 8, 0.5 μg (0.01 U). Molecular mass estimates (kDa) of enzyme activity bands are on the right.

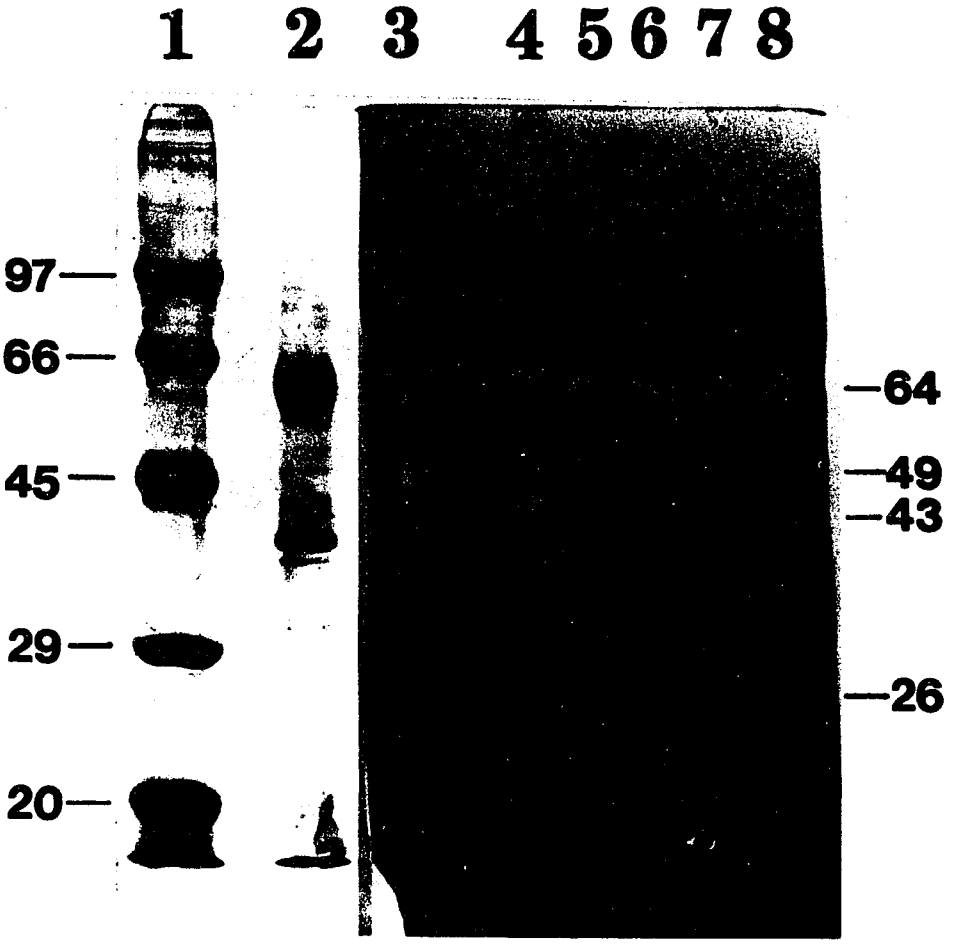

1 2 3 4 5 6 7 8
97
66
45
29
20
64
49
43
26

Table 6. Major cellulase components from *M. verrucaria* ATCC 9095 identified by zymogram (CMCase and MUCELase) analysis.

Molecular mass estimate (kDa)	CMCase[a]	MUCELase[b]
64	+	+
49	+	+
43	+	-
26	+	-

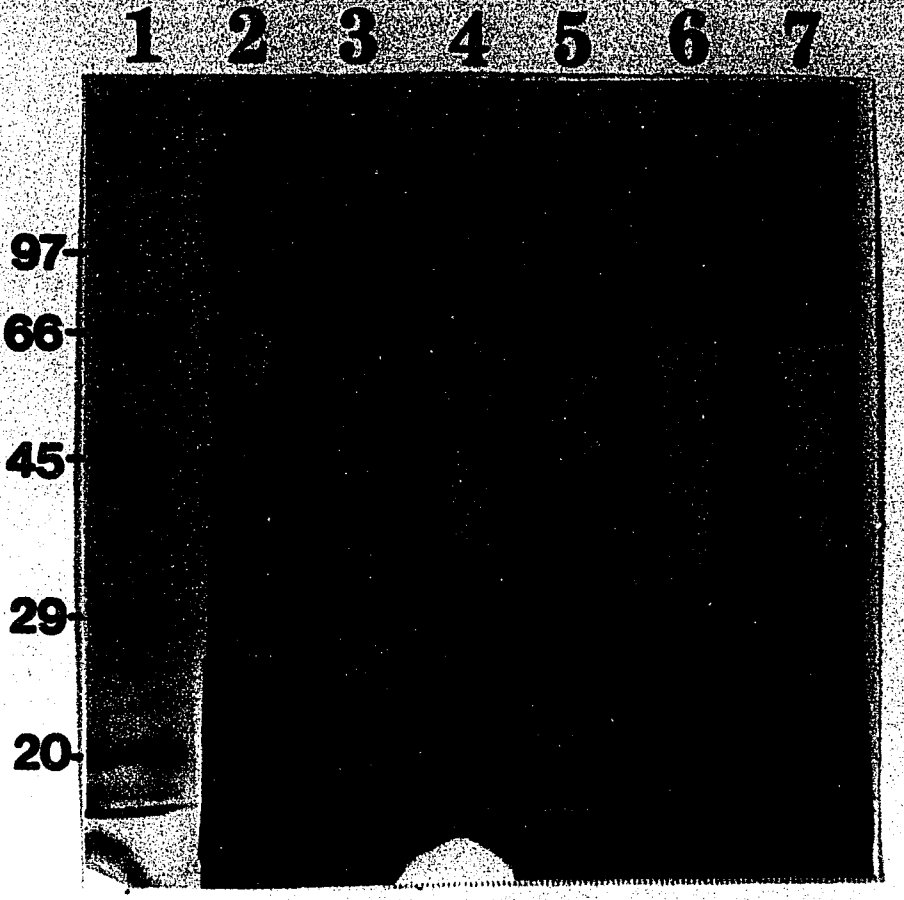
[a]CMCase activity was detected by staining a CMC incorporated gel with congo red after electrophoresis.

[b]MUCELase activity was detected by flooding a gel with MUCEL substrate solution after electrophoresis.

Comparison of zymogram activity (CMCase) patterns

CMCase activity patterns of *M. verrucaria* ATCC 9095,*T. reesei* QM9414, and preparations from 2 commercial cellulase sources are shown in Figure 5. Samples were denatured in 3% SDS at 95°C for 5 minutes and were subjected to 8% SDS-PAGE. All activity patterns displayed high molecular-mass components near the 97-kDa protein marker. The high molecular-mass component from *T. reesei* QM9414 (97 kDa) is more evident in Figure 1, lane 4 than in lane 3 of Figure 5. The most prominent CMCase bands appeared in the molecular mass range of 45 kDa to 60 kDa, although each cellulase source exhibited a different activity pattern. Activity patterns from the *M. verrucaria* ATCC 9095 (lane 2) and *T. reesei* QM9414 (lane 3) cellulase preparations displayed prominent low molecular-mass (<30 kDa) CMCase components, which were absent from the commercial sources (lanes 4-7). The commercial preparations displayed a relatively minor CMCase band below 20 kDa, which migrated with the buffer front (Figure 5, lanes 4 and 5; lanes 6 and 7). Three faint CMCase bands were observed at 38, 35, and 34 kDa from the *M. verrucaria* (lane 2) enzyme sample that were not evident in Figure 4. More protein sample was used in Figure 5, lane 2 (10 μg), than Figure 4, lane 4 (8 μg), to enhance CMCase band formation.

Figure 5. Comparison of zymogram (CMCase) activities in enzyme samples from *M. verrucaria* ATCC 9095, *T. reesei* QM 9414 and 2 commercial cellulase preparations. Samples were denatured in 3% SDS at 95°C for 5 minutes and were subjected to 8.0% SDS-PAGE. The separating gel contained 0.1% CMC. After electrophoresis, the gel was divided into 2 sections that consisted of lane 1 (molecular mass markers) and lanes 2-7 (enzyme samples). Lane 1 was silver-stained for protein. Values (kDa) of protein molecular mass markers in lane 1 are on the left. Lanes 2-7 were stained for CMCase activity with 0.1% congo red as described in "Materials and Methods". The following amounts of enzyme sample were used; Lane 2, 10.0 μg (0.3 U), *M. verrucaria* ATCC 9095; lane 3, 4.0 μg (0.4 U), *T. reesei* QM9414; lanes 4 and 5, 3.0 μg (0.5 U) and 1.5 μg (0.3 U), *T. reesei* Rohament CT; lanes 6 and 7, 3.0 μg (0.8 U) and 1.5 μg (0.4 U), *T. viride* Sigma Type V.


1 2 3 4 5 6 7
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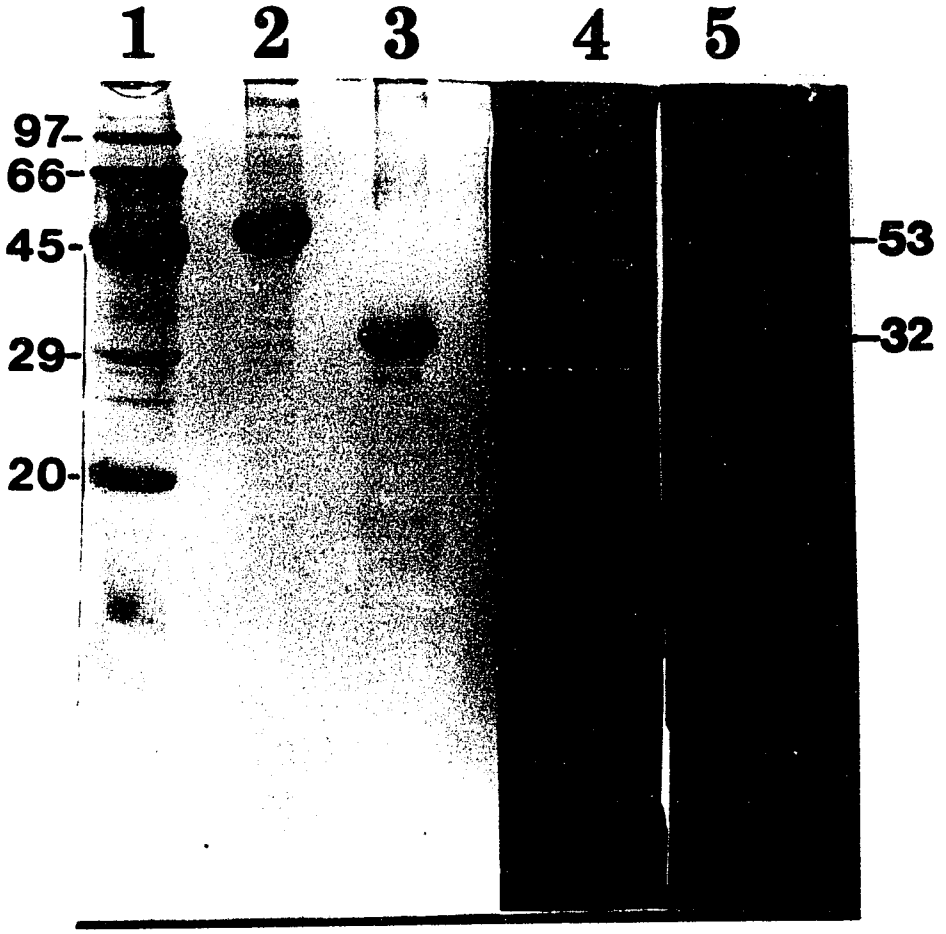
45
29
20

Zymogram analysis of the *B. subtilis* endoglucanases

CMCase zymogram analysis of two *B. subtilis* endoglucanase preparations (52.2 kDa and 30-33 kDa) are shown in Figure 6. Samples were denatured in 3% SDS at 95°C for 5 minutes and were subjected to 12% SDS-PAGE. Lanes 2 (52.2 kDa endoglucanase) and 3 (30-33 kDa endoglucanase) show the protein pattern of each endoglucanase preparation. The prominent CMCase bands were assigned molecular mass estimates of 53 kDa (lane 4) and 32 kDa (lane 5). A portion of the 32 kDa CMCase band in lane 5 was skewed during migration, although the molecular mass estimate was unaffected. Five minor CMCase bands (43, 40, 38, 35, and 32 kDa) were observed from the 52.2 kDa endoglucanase sample (lane 4). High molecular mass (>62 kDa) CMCase activity bands were present in both samples (lanes 4 and 5).

Figure 6. Zymogram (CMCase) analysis of two endoglucanase (EG) preparations derived from the same *B. subtilis* PAP115 gene. Samples were denatured in 3% SDS at 95°C for 5 minutes and were subjected to 12% SDS-PAGE. The separating gel contained 0.1% CMC. After electrophoresis, the gel was divided into 2 sections that consisted of lanes 1 (molecular mass markers), 2 (52.2 kDa EG), and 3 (30-33 kDa EG) and lanes 4 (52.2 EG) and 5 (30-33 kDa EG). Lanes 1-3 were silver-stained for protein. Values (kDa) of protein molecular mass markers in lane 1 are on the left. Lanes 4 and 5 were stained for CMCase activity with 0.1% congo red as described in "Materials and Methods". The following amounts of enzyme sample were used; Lane 2, 5 μg (3 U) and lane 4, 1 μg (0.6 U), 52.2 KDa endoglucanase; lane 3, 3 μg (2 U) and lane 5, 1 μg (0.7 U), 30-33 KDa endoglucanase. Molecular mass (kDa) estimates of CMCase activity bands are on the right.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DISCUSSION

Enzyme Production and Preparation

Crude extracellular cellulase preparations were prepared from *T. reesei* QM9414 and *M. verrucaria* ATCC 9095 for subsequent examination of endoglucanase isozymes using denaturing zymogram analyses. The liquid media used for cultivation of *T. reesei* QM 9414 and *M. verrucaria* ATCC 9095 were developed by Mandels and Andreotti (1978) and Whitaker (1953) especially for cellulase production by the fungi. In this study, Solka Floc (Mandels and Andreotti, 1978) and cotton linters (Whitaker, 1953) were replaced by cellulose from Sigma because the Sigma cellulose was a highly purified, uniform, and convenient carbon source. Cellulose is the most efficient inducer of cellulases (Messner et al., 1988). The enzyme preparations from *T. reesei* QM 9414 (Table 2) and *M. verrucaria* ATCC 9095 (Table 3) cannot be compared with those produced by Mandels and Andreotti (1978) and Whitaker (1953) because they used submerged culture methods and different enzyme assay protocols. *T. reesei* QM 9414 produced cellulase that was more active on CMC (4.8 U/ml *vs* 1.6 U/ml) and possessed a higher specific activity (68 U/mg *vs* 17 U/mg) than the *M. verrucaria* ATCC 9095 cellulase preparation. It has been reported that

Trichoderma reesei QM 6a produces cellulases that can more effectively degrade cotton fibers than cellulases from *M. verrucaria* QM 460 (ATCC 9095; Mandels and Reese, 1964). The reason why CMCase activity was not detected from the *M. verrucaria* culture fluid until day three is uncertain. Glucose can cause a decrease in cellulase production by *T. reesei* (Mandels and Andreotti, 1978); however, glucose reportedly stimulates cellulase synthesis by *M. verrucaria* (Hulme and Stranks, 1971).

In this study, culture filtrates from *M. verrucaria* and *T. reesei* were concentrated by lyophilization and desalted by using gel filtration. The greater increase in CMCase activity (12-fold) compared to protein concentration (7-fold for *T. reesei* and 7-fold for *M. verrucaria*) obtained after enzyme preparation could be attributed to removal of non-enzymatically active protein or enzyme inhibitors during culture filtrate processing. The increases in specific activity from 68 U/mg (Table 2) to 100 U/mg (Table 4) for *T. reesei* and from 17 U/mg to 29 U/mg for *M. verrucaria* could also be accounted for by the removal of non-enzymatically active protein or inhibitory materials. It was not necessary to concentrate the commercial cellulase preparations (*T. reesei*, Rohm Tech and *T. viride*, Sigma) because the quantity of protein in these preparations, 3.8 and 4.0 mg/ml, respectively, were sufficient for SDS-PAGE

and zymogram analyses.

The 52.2 kDa and 33 kDa *B. subtilis* endoglucanase samples, derived from the same endoglucanase gene, were purified by Dr. G. Willick (1991) and were recieved as ammonium sulfate precipitates. The 33 kDa endoglucanase sample was not desalted by ultrafiltration (10 kDa exclusion) because CMCase activity was detected in the ultrafiltrate (data not shown). Further concentration of the endoglucanase samples was not performed because the quantity of protein (0.6 mg/ml; Table 4) was sufficient for SDS-PAGE and zymogram analysis.

Zymogram Analyses

General conditions for zymogram analyses

The effects of denaturation temperature on activity (CMCase) pattern development were examined by treating enzyme samples at various temperature-time combinations in the presence of 3% SDS (final concentration) before SDS-PAGE. Since no differences in protein or activity (CMCase) patterns were observed (data not shown), a denaturation treatment of 5 minutes at 95°C was used for all subsequent zymogram analyses. dH_2O was used to wash separating gels instead of 0.1 M succinate buffer (pH 5.8) containing 10 mM dithiothreitol (Schwarz et al., 1987) because it was

convenient and had no detrimental effect on the CMCase zymogram patterns (data not shown).

Zymogram analyses of the *T. reesei* QM9414 cellulase preparation

A crude extracellular cellulase preparation from *T. reesei* QM9414 was subjected to denaturing SDS-PAGE and zymogram analyses to identify endoglucanase molecular forms. Reliability of the zymogram method was assessed by comparing molecular mass estimates assigned to the activity bands with molecular mass values of *T. reesei* endoglucanases reported in the literature. An enzyme sample from the *T. reesei* QM 9414 cellulase preparation was serially diluted and subjected to zymogram analysis to determine the number of distinct CMCase components (Figure 1, lanes 3-8). The CMCase components in Figure 1, lanes 3-8, were assigned molecular masses of 56, 44, 36, 34, 27, and 25 kDa. Based on CMCase zymogram analysis of a serially diluted enzyme sample, only a 56-kDa activity band was distinguishable (lanes 5-7) from the large, 45-58 kDa (lane 3), CMCase clearing zone.

Endoglucanase I, cellobiohydrolase I, and ß-glucosidase from *T. reesei* QM 9414 can all hydrolyze MUCEL (Deshpande et al., 1984). Endoglucanase (CMCase) zymogram activity bands that also possessed MUCELase activity (Figure 2, lane 5) were detected by the addition of cellobiose (5 mM) and

gluconolactone (1 mM) to the MUCEL substrate solution to inhibit cellobiohydrolase I and ß-glucosidase activities (Van Tilbeurgh and Claeyssens, 1985). The cellobiose (5 mM) and gluconolactone (1 mM) concentrations used were sufficient to inhibit 10 μg of purified cellobiohydrolase I and ß-glucosidase protein (Van Tilbeurgh and Claeyssens, 1985). Since only 5 μg of total protein was used for zymogram (CMCase and MUCELase) analysis (Figure 2, lanes 4 and 5), the inhibitor concentrations should have been sufficient to inhibit cellobiohydrolase I and ß-glucosidase. Cellobiohydrolase I holoenzyme has a molecular mass of 65 kDa and ß-glucosidase has a molecular mass of 73 kDa (Enari, et al., 1987). No fluorescent band was observed in the molecular mass regions for cellobiohydrolase I and ß-glucosidase in Figure 2, lane 4 (inhibitors were omitted from lane 4). The core fragment of cellobiohydrolase I is reported to have a molecular mass of 56 kDa, which is active on MUCEL (Van Tilbeurgh and Claeyssens, 1986). But pronounced core fragment formation seems to occur only during aggresive papain treatment on cellobiohydrolase I (Van Tilbeurgh and Claeyssens, 1986) or at a low pH (pH 4.0 or less) unbuffered culture medium high in peptone content (6 g/l; Mischak et al., 1989). The enzyme production medium used in this study was unbuffered (Mandels and Andreotti, 1978); however, the peptone concentration was low (0.5 g/l)

and the pH never decreased below 6.0 during cultivation (data not shown). Because no MUCELase activity band was detected in the molecular mass regions described for cellobiohydrolase I and ß-glucosidase in Figure 2, lane 4, the MUCELase activity observed in lane 5 (Figure 2) was attributed to an endoglucanase. Cellobiohydrolase I and ß-glucosidase may have been absent from the enzyme preparation or did not regain activity after the denaturation treatment. The appearence of a CMCase band (Figure 2, lane 4) corresponding to the 97-kDa molecular mass marker will be discussed under "comparison of zymogram (CMCase) activity patterns". Two endoglucanases have been cloned and sequenced from regulatory mutants of *T. reesei* QM9414 (Endoglucanase I, 55 kDa, Penttila et al., 1986 and Endoglucanase II, 50 kDa, Saloheimo et al., 1988). Both endoglucanases are active on CMC; however, only endoglucanase I gives a visable fluorescence reaction upon MUCEL hydrolysis. The 56-kDa zymogram band that was active on CMC and MUCEL in Figure 2 (lanes 5-7), was similar to the 56-kDa CMCase band in Figure 1 (lanes 5-7). The 56-kDa activity band displayed in Figure 1 (lanes 5-7), and Figure 2 (lane 5), may resemble endoglucanase I reported in a previous study (Penttila et al., 1986). Since MUCELase activity was not observed at the 49-kDa molecular mass region of Figure 2, lane 5, the large CMCase band observed

in the 44-58 kDa molecular mass region (Figure 2, lane 5) may be composed of at least one other endoglucanase that does not possess MUCELase activity. A distinct CMCase band in the 49-50 kDa molecular mass region could not be resolved by means of the gel concentrations used in Figures 1 (lane 3) or 2 (lane 5). The CMCase activity zone observed in the 49 kDa region (Figure 2, lane 5) may resemble endoglucanase II from *T. reesei* (Saloheimo et al., 1988). Several research groups (Grizali et al., 1979; Massiot, 1992; Messner et al., 1988; Niku-Paavola et al., 1985; and Stahlberg et al., 1988) have reported endoglucanases (36, 37, 38, and 43 kDa) in a molecular mass range similar to the three prominent 34, 36, and 44-42 kDa CMCase activity bands identified by zymogram analysis (Figure 1, lane 3 and Figure 2, lane 5). Recently, low molecular mass endoglucanases in the 25 kDa to 27 kDa range (Luderer et al., 1991; Massiot, 1992; Messner et al., 1988; Sprey and Uelker, 1992; and Uelker and Sprey, 1990) have been identified. These may resemble the low-molecular-mass CMCase species (24 kDa and 27 kDa) observed by using the zymogram protocol (Figure 2, lane 5). The 24 kDa CMCase component identified in Figure 2, lane 5, was assigned a molecular mass value of 25 kDa in Figure 1, lane 3. The faint CMCase bands (Figure 2, lane 4) observed at 28 kDa and at least two between 36-44 kDa are consistent with the molecular mass values of endoglucanases

reported by others (Messner et al., 1988; Stahlberg et al., 1988). The faint CMCase bands (28 kDa and 36-44 kDa) were most evident in Figure 2, lane 4, because the gel section was incubated for an additional 10 minutes at 45°C to accentuate band formation. A 67-kDa endoglucanase component, reported by two research groups (Messner et al., 1988 and Niku-Paavola et al., 1985), was not observed in my zymograms. Either the 67-kDa endoglucanase was inactivated during the denaturation treatment and did not renature, or it was never present in the enzyme preparation.

Endoglucanase I from *T. reesei* is considered a nonspecific endoglucanase because it possesses activity toward xylan as well as CMC and MUCEL (Beily, 1988). A zymogram analysis was performed to determine if the 56-kDa CMCase and MUCELase active component identified in Figure 2, lane 5, also possessed xylanase activity. The prominent xylanase activity band in Figure 3, lanes 2-7, was assigned a molecular mass of 55 kDa and resembled the shape of the CMCase and MUCELase band (56 kDa) identified as endoglucanase I in Figure 1, lanes 5-7, and Figure 2, lane 5. In addition, the xylanase activity pattern exhibited above 43 kDa was similar to the CMCase pattern (Figure 1, lane 3 and Figure 2, lane 5) displayed in the same molecular mass region. The appearence of a xylanase band near the 97 kDa molecular mass marker will be discussed under

"Comparison of zymogram activity patterns". It has been reported that endoglucanase II (50 kDa) does not have xylanase activity (Beily, 1988); however, prominent hydrolysis zones were evident in the 40-47 kDa region (Figure 3, lanes 2-6). Biely and Markovic (1988) demonstrated that at least six electophoretically different nonspecific endoglucanases produced by *T. reesei* were active on xylan. Dekker (1985) isolated a xylanase from *T. reesei* QM9414 that was 41 kDa in size. The xylanase activity bands in the 40-kDa to 47-kDa molecular mass region observed in Figure 3, lanes 2-7, could be caused by heretofore uncharacterized nonspecific endoglucanases or xylanases. Alternatively, endoglucanase II forms may exist that are active on xylan. Recently, two endoxylanases from *T. reesei* were isolated; their molecular masses were 20 kDa and 19 kDa (Tenkanen et al., 1992). The two xylanases may resemble the two prominent low molecular mass xylanase activity bands (Figure 3, lanes 2-6; 22 kDa and 20 kDa) that were identified by zymogram analysis. The five very faint xylanase bands observed between the 23 and 40 kDa molecular mass region may be enzyme components that possess slight activity toward xylan.

In general, the molecular mass values assigned to zymogram activity bands of *T. reesei* were consistent with the values of endoglucanases and xylanases previously

reported in the literature.

Several major concerns are associated with the zymogram method. First, only enzymes capable of surviving the denaturation-renaturation process will be detected. Second, additional research is neeeded to examine the possible generation of artifacts caused by incomplete denaturation. Although samples were heated at 95°C for 5 min in the presence of 3% SDS, reducing agents were omitted to avoid irreversible enzyme denaturation. Multisubunit endoglucanases have not yet been identified, but disulfide bonds are critical for maintaining enzyme compactness. Activity bands identified by zymogram analyses need to be isolated and the proteins sequenced to confirm their molecular masses.

Zymogram analysis of the *M. verrucaria* cellulase preparation

The only information on the fractionation of *M. verrucaria* ATCC 9095 cellulases was published by Selby and Maitland (1965). In their study, three cellulolytic components that possessed CMCase activity were identified from culture filtrates after separation by gel filtration chromatography. The molecular masses of the enzymes were 55, 30, and 5.3 kDa, respectively. The 55 and 30 kDa cellulases may resemble the 49 kDa and the 26 kDa CMCase bands identified by zymogram analysis (Figure 4, lanes 3-8). Selby and Maitland (1965) used Sephadex G-75 gel filtration

to separate the cellulase components. Resolution of the 43, 49, and 64 kDa CMCases identified by zymogram analysis would be difficult using Sephadex G-75, because the enzymes differed relatively little in molecular mass and were all active on CMC. The 55 kDa cellulase isolated by Selby and Maitland could actually be a mixture of several CMCases that had relatively similar molecular masses. Alternatively, the 43 and 64 kDa zymogram components were inadvertantly removed by Selby and Maitland (1965) during purification or were not present in their culture fluids. In this study, the culture filtrate was minimally processed to avoid enzyme loss or inactivation. MUCELase activties from *M. verrcaria* cellulase preparations have not been published. It seems that two CMCase species (65 kDa and 49 kDa) identified by zymogram analysis (Figure 3, lane 3) also possessed MUCELase activity. A 5.3 kDa CMCase reported by Selby and Maitland (1965) was undetected by zymogram analysis. The 5.3 kDa enzyme may have been irreversibly inactivated by the zymogram denaturation process or was absent from the enzyme preparation used in this study.

Comparison of zymogram (CMCase) activity patterns

Fungal endoglucanases with molecular masses greater than 70 kDa have been described by only a few investigators (Enari and Niku-Paavola, 1987). Messner et al. (1988) identified an SDS-stable 106-kDa endoglucanase yielding 58-

kDa components when the sample was treated with chloroform-methanol. Dominguez et al. (1992) recently demontrated that endoglucanase I can form aggregates in the pH range of 5.5 to 7.0. The high molecular mass (approximately 100 kDa) zymogram CMCase bands displayed by all the enzyme preparations in Figure 5, and the higher molecular mass xylanase activity band in Figure 3, lane 2, could be SDS-stable enzyme multimers or aggregates similar to those described by Messner et al. (1988) and Dominguez et al. (1992). The high molecular mass (97 kDa) CMCase displayed by *T. reesei* QM 9414 is most evident in Figure 2, lane 4. Most fungal endoglucanases are between 30 and 60 kDa in size (Enari and Niku-Paavola, 1987). This finding coincides with the activity patterns displayed in Figure 5. Low molecular mass endoglucanases (<30 kDa) have been reported from *M. verrucaria* (Selby and Maitland, 1965), *T. reesei* (Sprey and Uelker, 1992), and a *T. viride* commercial enzyme preparation (Berghem et al. 1976). Prominent low-molecular-mass CMCase bands from the commercial *T. reesei* (Rohm Tech) enzyme preparation (Figure 5, lanes 4 and 5) were either absent in the original sample or irreversibly inactivated by the zymogram denaturation treatment. The latter possibility is unlikely because low molecular mass CMCase species were detected in the *T. reesei* QM9414 cellulase preparation (Figure 5, lane 3). Inconsistencies in the endoglucanase

composition in *T. reesei* cellulase preparations are believed to be attributed to variations in enzyme production and in purification methods (Enari and Niku-Paavola, 1987). Messner et al. (1988) demonstrated that endoglucanases from *T. reesei* depended upon the cellulase-inducing carbon source used for cultivation. In particular, a low-molecular-mass (<30 kDa) endoglucanase was absent when lactose, rather than cellulose, was used as the carbon source. Using monoclonal antibodies against endoglucanase I, Kubicek-Pranz et al. (1991) characterized 15 cellulase preparations from *T. reesei* by SDS-PAGE and immunoblotting. Most samples exhibited a 55-kDa band which was characteristic of endoglucanase I, but other molecular forms of the enzyme were evident. Activities of the endoglucanase forms could not be determined by using the immunoblot method. *In situ* zymogram analysis may complement immunoblotting studies by directly detecting activities (CMCase) of different endoglucanase forms and also by providing information on cellulase composition.

The low-molecular-mass *T. viride* CMCase band (Figure 5, lane 7) that migrated with the buffer front may be the same enzyme (12.5 kDa) isolated from a commercial cellulase preparation of *T. viride* (Berghem et al., 1978). An endoglucanase below 20 kDa has not been reported from *T. reesei*.

The faint CMCase bands (38, 35, and 34 kDa) observed from the *M. verrucaria* enzyme sample in Figure 5, lane 2, that were not evident in Figure 4, probably were minor endoglucanase components that could not be detected by the methods used in the Selby and Maitland (1965) investigation.

Zymogram analysis of *B. subtilis* endoglucanases

Two cloned and purified *B. subtilis* endoglucanases with known molecular masses of 52.2 and 30-33 kDa were subjected to zymogram analysis to determine the reliabilty and compatibility of the technique (Figure 6). The 52.2-kDa endoglucanase is reportedly secreted into the culture medium as a 52.2-kDa proenzyme and is progressively cleaved to a 32-kDa protein (Lo et al., 1988). The activity of each cleaved protein fragment was not specifically determined, but total CMCase activity in the culture medium did not decrease during the course of the study (Lo et al., 1988). The five minor CMCase bands (Figure 6, lane 4) could be active components from processed 52.2 kDa proenzyme. The high-molecular-mass (>62 kDa) zymogram CMCase bands observed in both samples may be similar to the SDS-stable multimeric enzyme components described by Messner et al. (1988).

In general, the molecular mass estimates assigned to the prominent CMCase bands from both endoglucanase samples in Figure 6, lanes 4 (53 kDa) and 5 (32 kDa) were consistent with the reported values (52.2 and 30-33 kDa).

GENERAL SUMMARY

PART I describes development of a screening procedure to detect low molecular mass (<10 kDa) enzymes from microorganisms, commercial enzyme preparations and mammalian tissue samples. Samples were subjected to ultrafiltration by using a 10-kDa exclusion membrane and enzyme activities in the ultrafiltrates and retentates were determined by radial diffusion in substrate-laden agar media. Passage tests of proteins with known molecular masses revealed that cytochrome c (12.5 kDa) and *Chainia* sp. xylanase (about 5 kDa) passed through ultrafilters with a 10-kDa membrane exclusion, but larger proteins did not.

One hundred sixty-five bacterial isolates, 79 fungal strains, 10 commercial preparations, and 3 mammalian liver tissue samples were examined for the presence of small enzymes. 4-Methlyubelliferyl-phosphate and CMC were the most frequent substrates degraded by bacterial and fungal isolates. Six fungal species, including *A. niger* ATCC 9029, eight unidentified fungal isolates, and two commercial fungal enzyme preparations possessed CMCase and xylanase activities that penetrated ultrafilters with a 10 kDa exclusion. A purified endoglucanase from *B. subtilis* PAP 115, with a molecular mass between 30-33 kDa, also was ultrafiltrate-positive for CMCase activity.

Zymogram analysis of the *A. niger* ATCC 9029 enzyme preparation revealed that a xylanase component with a molecular mass of 23 kDa passed through the ultrafilter membrane. In accordance with other studies (Grabski and Jeffries, 1991; Wong et al., 1986), the 23-kDa xylanase may have a compact structure that allows passage through a membrane with a 10 kDa exclusion. Alternatively, nonuniform pore sizes in the ultrafilter membrane could account for enzyme activity detected in the ultrafiltrate, although this latter explanation is unlikely (refer to discussion on page 56). Zymogram (CMCase) analysis of the *B. subtilis* endoglucanase sample confirmed that a CMCase component of 30 kDa passed through a membrane with a 10 kDa exclusion. The ability of the *B. subtilis* PAP115 endoglucanase to penetrate a 10 kDa exclusion ultrafilter may be by a mechanism similar to that described for the 23-kDa xylanase reported in this study.

The screening procedure successfully detected CMCases and xylanases that penetrated an ultrafilter with a 10 kDa exclusion; however, no small enzymes were found that could be used as labels for diagnostic probes.

In PART II, the Schwarz et al. (1987) *in situ* zymogram method was adapted for the identification of endoglucanase molecular forms produced by mesophilic fungi (*M. verrucaria* ATCC 9095 and *T. reesei* QM9414), *B. subtilis* PAP115, and two

different commercial-enzyme preparations.

Extracellular cellulase preparations from *M. verrucaria* ATCC 9095 and *T. reesei* QM9414 were produced by shake flask culture, freezed-dried, and desalted by gel filtration. By using these methods, sufficient quantities of enzyme were produced and concentrated for their subsequent examination by SDS-PAGE and zymogram analyses. No apparent difference in the CMCase activity pattern or protein profile was observed when *M. verrucaria* ATCC 9095 and *T. reesei* QM 9414 enzyme samples were denatured at temperatures ranging from 60°C to 95°C in the presence of SDS. In general, the molecular mass values assigned to zymogram activity bands (CMCase and MUCELase) correlated well with the values of endoglucanases previously identified from *M. verrucaria* ATCC 9095 and *T. reesei*. The presence of several CMCase zymogram bands (67 kDa and 44 kDa) from *M. verrucaria* ATCC 9095 that were not detected by Selby and Maitland (1964) could be a result of the different enzyme preparation and separation methods used. A 5.3 kDa CMCase identified by Selby and Maitland (1964) was undetected by zymogram analysis. The latter CMCase was either inactivated by the SDS-PAGE denaturation treatment or was absent from the enzyme preparation. CMCase and xylanase zymogram bands in the 42-kDa to 58-kDa region that were identified from *T. reesei* QM9414 seemed to contain several enzyme components with

slight differences in molecular mass. A comparison of CMCase activity patterns from *M. verrucaria*, *T. reesei*, and two commercial-cellulase preparations revealed that all samples displayed high-molecular-mass (near the 97-kDa marker) CMCase components. The high-molecular-mass CMCases were probably SDS-stable multimers of lower-molecular-mass endoglucanases. The molecular mass values of prominent zymogram CMCase bands (53 kDa and 32 kDa) from *B. subtilis* endoglucanases were consistent with the reported values (52.2 kDa and 30-33 kDa). The 52.2 kDa endoglucanase sample also displayed five minor CMCase components after zymogram analysis. The minor CMCases ranged from 43 kDa to 32 kDa in size and probably resulted from proteolytic processing of the 52.2-kDa endoglucanase.

Zymogram analysis after denaturing SDS-PAGE was a reliable and simple method for determining enzyme mass (PART I) and identifying endoglucanase and xylanase molecular forms (PART II). The zymogram technique would aid studies involving endoglucanase induction and processing, preliminary characterizatiion of new endoglucanase systems, and screening for specific endoglucanase properties (e.g. specificity and molecular mass).

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